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DIAGNOSTIC AND PROTECTIVE ANTIGEN GENE SEQUENCES OF ICHTHYOPHTHIRIUS

This application claims the benefit of U.S. Provisional Application Serial No. 60/131,121, filed April 27, 1999; U.S. Provisional Application 60/118,634, filed February 4, 1999; U.S. Provisional Application 60/122,372, filed March 2, 1999; and U.S. Provisional Application 60/124,905, filed March 17, 1999, each of which is incorporated herein by reference in its entirety.

Cross Reference to Related Applications

This application incorporates the content of U.S. Patent Application Serial No. 09/498,612, entitled "Recombinant Expression of Heterologous Nucleic Acids in Protozoa," filed February 4, 2000.

Statement of Government Rights

This invention was made with government support under grants from the United States Department of Agriculture (USDA) CSRS NRICGP, Grant No. 95-37204-2139. The U.S. government has certain rights in this invention.

Background of the Invention

Ichthyophthirius multifiliis is a holotrichous ciliated protozoan which is an obligate parasite of freshwater fish. The life cycle of the parasite includes a free-living infectious stage (the theront or tomite) and an obligate fish-associated feeding stage (the trophont or trophozoite). The infective theront invades the skin and the gill epithelia, resulting in disturbances in respiratory and excretory functions. Once in epithelial tissue, the theront differentiates into the fish-associated feeding form known as a trophont. When the trophont is mature, it is

released from the surface of the fish and secretes material forming a gelatinous cyst. Within the cyst, the mature trophont undergoes multiple cell divisions to produce hundreds of theronts, which are then released from the matrix to begin a new cycle of infection.

Protection from Disease Caused by I. multifiliis

Ichthyophthiriasis, the disease caused by this parasite, is commonly referred to as "Ich" or "white spot disease." Under conditions of intensive aquaculture, Ich frequently has a high morbidity and mortality, resulting in significant financial losses to fish producers.

Treatments are available for Ich-infected fish. However, the chemical treatments are effective only against the free-living theronts; there is no known agent for eliminating trophonts associated with the host. Furthermore, some of the chemotherapeutic agents used to treat Ich are suspected to leave residues in treated fish and to be carcinogenic. As a result, certain of the available treatments, e.g., malachite green, are not permitted for fish raised for human consumption. In addition, chemical treatments result in physiological stress to the infected fish beyond that resulting directly from the infection.

Those fish which survive infection by *I. multifiliis* are generally immune to further infection by the live parasite. Early reports suggested that fish (mirror carp) were successfully immunized by exposure to sublethal doses of the parasite (Hines et al., J. Fish. Biol. 6:373-378 (1974)) and by exposure to the live parasite in conjunction with chemical treatment. There have also been reports of at least partial protective immunity in fish vaccinated using the killed parasite. For example, a substantial decrease in the number of infective parasites on the body surface of goldfish which had been previously injected with killed theronts was observed when the fish were challenged with a measured dose of live theronts (Parker, Studies on the Natural History of *Ichthyophthirius multifiliis* Fouquet 1876, an Ectoparasitic Ciliate of Fish, Ph.D. Dissertation, The University of Maryland, College Park, Maryland (1965)). Areerat ("The Immune Response of Channel Catfish, *Ictalurus punctatus* (Rafinesque), to *Ichthyophthirius*

multifiliis", unpublished Master's thesis, Auburn University) reported that channel catfish injected with formalin-fixed trophonts were protected when challenged with a lethal dose of infective theronts. Goven et al. (J. Fish Biol. 17:311-316 (1980)) reported initial protection against lethal infection when fish were injected intraperitoneally with theront cilia. The experiment was discontinued when all control fish had died and the fate of the vaccinated fish was not followed further.

More recently, however, Burkhardt et al. (J. Fish Dis. 13:401-410 (1990)) reported that neither immersion exposure nor intraperitoneal injection with killed *I. multifiliis* theronts conferred protective immunity to challenge doses of live theronts, although there was a delay in mortality of the vaccinated fish was observed. Likewise, intraperitoneal injection with theront cilia preparations did not prevent mortality, only delayed it. Only intraperitoneal injection with live theronts was effective in preventing mortality after challenge with infective parasites. Those fish which had been injected with live theronts remained protected against infection for an extended time, as evidenced by their resistance to challenge infections at 3 and 13 months after the original injection. Attempted immunization with formalin-fixed trophonts led to some delay in mortality but had an unclear effect on ultimate mortality.

The mucus coating of an immune fish participates in protection from Ich infection. Hines et al. (J. Fish. Biol. 6:373-378 (1974)) showed that both sera and mucus from immune fish was capable of immobilizing the infective form of *I. multifiliis*. These authors also noted that fish recovering from Ich had a different distribution of the parasite than did newly infected fish. Newly infected fish exhibited parasites all over the body while a recovering fish exhibits parasites primarily at edges of the fish. These are the parts of the fish which are least well supplied with blood, and therefore, would be less well supplied with antibodies.

Clark et al. (Devel. Comp. Immun. 1--2:581-594 (1988)) studied the sera of channel catfish that had been rendered immune to further Ich infection by exposure to sublethal surface infection and treated with chemotherapy. The sera

of these immune catfish contain antibodies which specifically bind to *I. multifiliis* cilia; little cross-reactivity was observed for cilia prepared from the free-living ciliate *Tetrahymena thermophila*. Whole *I. multifiliis* cilia and a ciliary membrane fraction gave similar reactions with the immune sera, but axoneme fractions showed little differential reaction in comparisons between immune and preimmune sera. However, attempts to identify the ciliary proteins with which the antibodies reacted using blots from SDS gel electropherograms were not successful. Sera from immune fish also immobilize the parasite *in vitro*, with an apparent positive correlation between specific antibodies and immobilization of theronts *in vitro*.

There were early reports that fish vaccinated with *Tetrahymena pyriformis* and with *T. thermophila* or with cilia prepared from *Tetrahymena* were protected from Ich infection (e.g., U.S. Pat. No. 4,309,416, ~~Dawe et al.~~ Grateck et al). It had been proposed that the ciliary membrane antigens from *Tetrahymena* showed cross-reactivity with those of *I. multifiliis*. However, more recent reports showed that attempted vaccination of channel catfish with *T. thermophila* Lwoff cilia did not protect the fish from subsequent challenge with *I. multifiliis* (Burkhardt et al., J. Fish Dis. 13:401-410 (1990)). It has been postulated that the previous cross-reactivity was due to the conserved axoneme proteins, rather than due to serologically related ciliary membrane proteins.

I. multifiliis i-antigens

A novel mechanism of humoral immunity against *I. multifiliis* was recently described. Rather than being killed on the host, a majority of parasites are forced to exit fish prematurely in response to antibody binding (M. Cross, J. Fish Dis., 15:497-505 (1992))(T. Clark et al., Parasitol. Today, 13:477-480 (1997)). While the precise mechanism underlying this phenomenon is unknown, the target antigens responsible for premature exit have been identified as a class of abundant surface membrane proteins known as immobilization antigens, or i-antigens (T. Clark et al., Annu. Rev. Fish Dis., 5:113-131 (1995)). Antibodies against these proteins rapidly immobilize cells *in vitro*.

I-antigens are common to a variety of hymenostomatid ciliates and have been intensively studied in *Paramecium* and *Tetrahymena* where their expression undergoes marked variation in response to environmental stimuli (F. Caron et al., Annu. Rev. Microbiol., 43:23-42 (1989); Smith et al., J. Protozool., 39:420-428 (1992)). Antigenic switching in these cells results from the differential expression of multiple i-antigen genes under defined sets of conditions and represents one of the most striking examples of antigenic shift in nature. To date, there is little evidence that this type of variation occurs in *Ichthyophthirius*; however, steady-state levels of i-antigen transcripts vary as much as 50 fold during transition from the host-associated trophont to the infective theront stage, and it is clear that the genes for these proteins are developmentally regulated through the parasite life cycle (T. Clark et al., Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)). Furthermore, serotypic variants of the i-antigens have been described among geographic isolates of the parasite (H. Dickerson et al., J. Euk. Microbiol., 40:816-820 (1993)).

Although i-antigens have gained considerable attention with regard to their mode of expression, their biological function remains obscure. In *Paramecium* and *Tetrahymena,* i-antigens are linked to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor, and in some cases, form a thick layer that coats the plasma and ciliary membranes (F. Caron et al., Annu. Rev. Microbiol., 43:23-42 (1989)). This has led to speculation that their primary function is to shield the cell membrane from environmental insult; indeed, this fits a general model for the role of GPI-anchored proteins in lower eukaryotes. The fact that cross-linking of i-antigens at the surface of *Ichthyophthirius* elicits a physiological response in the parasite also suggests that these proteins may play a role in transmembrane signaling (T. Clark et al., Parasitol. Today, 13:477-480 (1997)). Consistent with this idea, i-antigen antibodies trigger mucocyst discharge in both *I. multifiliis* (T. Clark et al., J. Fish Biol., 31(A):203-208 (1987)) and *Tetrahymena thermophila* (J. Alexander, Trans. Amer. Microsc. Soc., 86:421-427 (1967)), as well as trichocyst discharge in *Paramecium* ssp.

The cDNA sequence associated with a 48 kD i-antigen from an isolate of *I. multifiliis* (G1 isolate, serotype A) was reported by Clark et al. (Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)); serotyping was reported by Dickerson et al. (Annu. Rev. Fish Dis. 6:107-120 (1996)). A recombinant subunit vaccine derived from this cDNA sequence was reported by He et al. (Aquaculture 158: 1-10 (1997)). This subunit vaccine was engineered as a recombinant glutathione sulfotransferase (GST) fusion with a 105 amino acid fragment of the protein that the researchers identified as a potential antigenic epitope, corresponding to one of several tandemly repetitive amino acid sequence domains identified by Clark et al. (Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)). The nucleotide sequence encoding the fusion construct was chemically synthesized and used for expression of the recombinant peptide in bacteria. Two amino acid substitutions relative to the native sequence were required in order to provide restriction sites in the corresponding DNA; moreover, protozoan glutamine codons TAA and TAG (which function as stop codons in *E. coli* and other conventional protein expression systems) were replaced by the universal glutamine codons CAG or CAA in order to allow expression of the fusion construct in *E. coli*. The fusion vaccine gave weak protection against an undefined isolate of *I. multifiliis*; 50% of the vaccinated fish were heavily infected with *I. multifiliis* upon challenge with the live parasite, compared to 75% of control fish.

The 48 kD i-antigen protein has been isolated from cultures of *I. multifiliis* (Clark et al., Annu. Rev. Fish Dis. 5:113-131 (1995); Lin et al., J. Protozoology 39:457-463 (1992)). In addition, a 55 kD i-antigen protein has been isolated from cultures of *I. multifiliis* and affinity purified and used in studies on passive immunity (T. L. Lin et al., Inf. Immun. 64:4085-4090 (1996)). Mouse monoclonal antibodies raised against this protein were effective to immobilize G5 isolates of *I. multifiliis*. However, a native i-antigen protein would be very difficult to obtain from *I. multifiliis* in large quantity because this obligate parasite cannot be easily cultured.

It is clear that an inexpensive and effective vaccine against Ich would be of great benefit to the aquaculture industry.

Summary of the Invention

The present invention is directed to novel i-antigen polypeptides and nucleic acid molecules that encode them. Examples of novel i-antigen polypeptides include polypeptides having SEQ ID NO:6 and SEQ ID NO:7 derived from *I. multifiliis*. The nucleic acid molecule provided by the invention contains a polynucleotide fragment having a nucleotide sequence that encodes at least a portion of an i-antigen polypeptide, exemplified by nucleotide sequences SEQ ID NOs: 1, 3 and 5. In one embodiment, the polynucleotide fragment encodes at least a C-terminal portion of an i-antigen polypeptide having amino acid sequence SEQ ID NO:6; in another it encodes at least one terminal portion of an i-antigen polypeptide having amino acid sequence SEQ ID NO:7; in yet another it encodes at least an antigenic portion an i-antigen polypeptide having amino acid sequence SEQ ID NO:7. The nucleic acid molecule of the invention can take the form of a vector that is capable of expression in an organism or in a cell, preferably a fish and/or in a conventional protein expression system, including bacteria, such as *E. coli*, yeast, mammalian cell culture or insect cells. The invention includes a host cell transformed with the vector of the invention, and further includes an organism, preferably a fish, comprising a nucleic acid molecule of the invention. Also included is an antibody capable of binding at least one of the i-antigen polypeptides of the invention.

The invention further includes a composition for inducing an immune response in a fish comprising a nucleic acid molecule that has a nucleotide sequence that encodes an antigenic portion of an i-antigen polypeptide of the invention. Likewise, the invention includes a composition for inducing an immune response in a fish comprising an antigenic i-antigen polypeptide of the invention. The invention further includes a method for causing an immune response in the fish by administering an immunogenic composition as described, for example as a prophylactic or therapeutic vaccine. The composition preferably prevents or controls *I. multifiliis* infection in fish.

Also included in the invention is a method for detecting *Ichthyophthirius* in an aquaculture. A sample containing nucleic acid is obtained from an aquaculture fish or from water present in the aquaculture, then at least one primer oligonucleotide having a sequence complementary to at least a portion of SEQ ID NO:6 or SEQ ID NO:7 is added to the nucleic acid sample. A polymerase chain reaction amplification is conducted, and the amplified product is analyzed for the presence of a product amplified by the at least one oligonucleotide primer. Advantageously, the amplified product can then be used to formulate or customize a vaccine effective to treat or prevent *Ichthyophthirius* infection. The method thus optionally includes making a polynucleotide vaccine that contains at least a portion of the amplified product, or a protein subunit vaccine that includes an antigenic polypeptide encoded by the portion of the amplified product; and administering the vaccine to treat or prevent *Ichthyophthirius* infection in a fish or fish population.

Further, the invention provides a method for identifying an *I. multifiliis* serotype. A sample containing *I. multifiliis* nucleic acid is combined with at least one primer oligonucleotide that has a sequence complementary to a region of an *I. multifiliis* nucleotide sequence encoding an i-antigen that is unique to and selective for that serotype, then a polymerase chain reaction amplification is conducted. The reaction mixture is analyzed for the presence of a product amplified by the serotype-selective oligonucleotide primer.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence (SEQ ID NO:2) and the deduced amino acid sequence (SEQ ID NO:6) of the 48 kD i-antigen gene (*IAG48[G1]*); a guanine (G) nucleotide that marks the start of the 1.2 kb cDNA is indicated by the arrow; the adenosine (A) nucleotide in the gene's putative ATG start codon for the 48 kD i-antigen is assigned the number +1; a stretch of 14 mostly hydrophobic amino acids at the C-terminus of the deduced protein is boxed; the 3 small amino acids (Cys-Ala-Ser, denoted with asterisks) may represent the site at which cleavage and GPI-anchor addition occurs.

Figure 2 shows nucleotide sequences for (a) the native G5 55 kD i-antigen coding region including stop codons (SEQ ID NO:44); and (b) a synthetic 55 kD i-antigen coding region, including stop codons (SEQ ID NO:102), useful as a DNA vaccine and in conventional protein expression systems.

Figure 3 shows (a) an alignment of the deduced amino acid sequences of the genomic 48 kD (upper line) (SEQ ID NO:6) and 55 kD (lower line) (SEQ ID NO:7) i-antigens of *I. multifiliis*; where asterisks indicate identities between the two deduced protein sequences, double dots indicate highly homologous amino acids, and single dots indicate moderately homologous amino acids; boxes indicate conserved regions; and (b) an alignment of the nucleotide sequences of the coding regions of the *IAG48 [G1]* gene (upper line) (SEQ ID NO:1) and the *IAG55 [G5]* gene (lower line) (SEQ ID NO:3) of *I. multifiliis*, where asterisks indicate identities between the two nucleotide sequences.

Figure 4 shows the deduced amino acid sequence of the 55 kD i-antigen encoded by the *IAG55 [G5]* coding region in Fig. 2(a).

Figure 5 shows an amino acid sequence alignment of five homologous tandemly repeated amino acid sequence domains of (a) the deduced 48 kD i-antigen genomic coding region and (b) the deduced 55 kD i-antigen coding region; amino acids that are shared by three or more repeats are boxed; asterisks denote cysteine residues conserved in all repeats.

Figure 6 is a restriction map of the cloned genomic DNA fragment encoding the 48 kD i-antigen; *Swa* I, (S); *Eco*R I, (E); *Nsi*, (N); and *Hind* III, (H); the filled portion indicates the coding region of the i-antigen gene.

Figure 7 shows a comparison between the *IAG48 [G1]* gene sequence and 1.2 kb cDNA sequence. Panel (A) is a schematic illustrating the basic differences between the gene and cDNA sequences (boxed); the filled regions of the gene sequence are not present in the 1.2 kb cDNA sequence; ATG, start codon; TGA, stop codon; nt, nucleotides; inverted triangle indicates the site of the observed C/T transversion. Panel (B) diagrams equivalent regions of the 1.2 kb cDNA (nucleotides 1-1172) and *IAG48 [G1]* gene (nucleotides 56-1227); the

C/T transversion is indicated at nucleotide 897 (cDNA) and 952 (genomic DNA). Panel (C) shows the nucleotide sequences specified by the 3' ends of the 1.2 kb cDNA (SEQ ID NO:64), the *IAG48 [G1]* gene (SEQ ID NO:66), the ICH5/EPB 3' RACE product (SEQ ID NO:67), and two additional cDNAs designated 1-3 (SEQ ID NO: 68) and 1-1 (SEQ ID NO:69); also shown are the corresponding deduced amino acid sequences of the 1.2 kb cDNA (SEQ ID NO:63) and the *IAG48 [G1]* gene (SEQ ID NO:65); a point mutation in the RACE product is boxed and is most likely attributable to the use of a low fidelity thermostable DNA polymerase during synthesis; the bracket covering nucleotides +1409 through +1413 in the *IAG48[G1]* transcript indicates a putative polyadenylation site.

Figure 8 shows an amino acid sequence alignment of *I. multifiliis* and *Giardia* surface proteins; the *IAG48[G1]* (amino acids 20-428) (SEQ ID NO:61) and *Giardia lamblia* vspA6-S1 (amino acids 61-459) (SEQ ID NO:62) gene products were compared using CLUSTALW multiple sequence alignment software; common cysteine residues are boxed; the segment of the *Giardia* VSP shown here (accession no. Q24970) comprises 67% of the predicted protein.

Figure 9 is a schematic illustrating the method used to isolate the 1 kb cDNA encoding a portion of the G5 55 kD i-antigen sequence.

Figure 10 is a schematic illustrating a method utilizing inverse PCR that was used to obtain the nucleotide sequences flanking the 1 kb cDNA sequence in the gene encoding the G5 55 kD i-antigen.

Figure 11 shows an SDS-PAGE gel and a Western blot of solubilized and GPI-phospholipase C treated membrane proteins from *I. multifiliis* (strain G5).

Figure 12 lists oligonucleotide primers used for DNA shuffling-based synthesis of a G5 synthetic i-antigen gene.

Figure 13 shows the nucleotide sequence encoding the synthetic G5 proline mutant i-antigen protein (L6P) (SEQ ID NO:53); the arrow indicates the mutation position.

Figure 14 shows the amino acid sequence of a synthetic G5 proline mutant i-antigen protein (L6P) (SEQ ID NO:54); the arrow indicates the position of the mutation.

Figure 15 is a Western blot showing expression of a recombinant synthetic 55 kD i-antigen protein in *E. coli*.

Figure 16 shows survival and days to death of channel catfish vaccinated with purified 55 kD i-antigen subunit protein.

Figure 17 is a Western blot showing expression and secretion from a transformed *Tetrahymena* of a G1 i-antigen with C-terminal truncation.

Figure 18 shows Western blotting analysis of sera from fish vaccinated with the 55 kD plasmid vaccines.

Figure 19 shows ELISA results for fish vaccinated with *Tetrahymena* expressing (a) membrane associated G1 i-antigen and (b) secretary form of G1 i-antigen.

Figure 20 is a 10X magnification of serotype A *I. multifiliis* an immobilization test conducted using sera (1:20 dilution) from (a) fish vaccinated with live *Tetrahymena* expressing *neo* ("anti-live Tneo," the negative control) and (b) fish vaccinated with live *Tetrahymena* expressing the full-length 48 kD i-antigen protein from a G1 *I. multifiliis* isolate ("anti-live TG1).

Figure 21 is a gel showing the use of universal primers P2 and P4 to amplify nucleotide sequences in several different *I. multifiliis* serotypes.

Detailed Description of the Invention

The present invention provides a nucleic acid molecule comprising a polynucleotide fragment having a nucleotide sequence that encodes at least a portion of an i-antigen protein.

In one aspect of the invention, the nucleotide sequence of the polynucleotide fragment encoding the i-antigen protein is derived from *I. multifiliis* (G1 isolate), and encodes a putative i-antigen having a molecular weight of about 48 kD. In a preferred embodiment of this aspect of the invention, the polynucleotide fragment is at least about 20 nucleotides in length

and has at least a 3' terminal portion of a nucleotide sequence as shown in Fig. 1, nucleotides 1 through 1326 (SEQ ID NO:1), wherein nucleotides 1 through 1326 represent the coding region of the *IAG48[G1]* gene of *I. multifiliis*. In a particularly preferred embodiment, the polynucleotide fragment is SEQ ID NO:1. The term "3' terminal portion" of SEQ ID NO:1 includes at least one nucleotide contiguous to and located 3' of nucleotide 1226, exemplified by nucleotides 1227 through 1326 (SEQ ID NO:4) in SEQ ID NO:1. A "coding region" is a linear string of nucleotides that encodes a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. Optionally, the polynucleotide fragment of the invention includes one or more flanking nucleotides on one or both of the 5' and 3' ends of the coding region, representing untranslated regions (UTRs) as shown in Fig. 1. A polynucleotide fragment of the invention can, for example, have a nucleotide sequence represented by nucleotides -432 through 2054, as shown in Fig. 1 (SEQ ID NO:2).

In another aspect of the invention, the nucleotide sequence of the polynucleotide fragment encoding an i-antigen protein is derived from *I. multifiliis* (G5 isolate), and encodes an i-antigen having a molecular weight of about 55 kD. In a preferred embodiment of this aspect of the invention, the polynucleotide fragment is at least about 20 nucleotides in length and has at least one terminal portion of a nucleotide sequence as shown in Fig. 2(a), nucleotides 1 through 1404 (SEQ ID NO:3), wherein nucleotides 1 through 1404 represent the coding region of the *IAG55[G5]* gene of *I. multifiliis*. A terminal portion of SEQ ID NO:3 can be a 5' terminal portion or a 3' terminal portion of SEQ ID NO:3. A 5' terminal portion of SEQ ID NO:3 includes at least one nucleotide contiguous to and located 5' of nucleotide 61, exemplified by nucleotides 1 through 60 (SEQ ID NO:19) in Fig. 2(a). Likewise, a 3' terminal portion of SEQ ID NO:3 includes at least one nucleotide contiguous to and located 3' of nucleotide 1344, exemplified by nucleotides 1345 through 1404 (SEQ ID NO:20) in Fig. 2(a). In a particularly preferred embodiment, the polynucleotide

fragment is SEQ ID NO:3. Optionally, the polynucleotide fragment of the invention includes one or more flanking nucleotides on one or both of the 5' and 3' ends of the coding region.

The invention further includes a nucleic acid molecule comprising a polynucleotide fragment having a nucleotide sequence selected from the class of nucleotide sequences that encodes a polypeptide having at least a C-terminal portion of SEQ ID NO:6 (Fig. 1). Preferably, the polynucleotide is at least about 20 nucleotides in length. The term "polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, and protein are included within the definition of polypeptide. A "C-terminal portion" of SEQ ID NO:6 includes at least one amino acid contiguous to and located to the C-terminal side of amino acid 409 (Ala409) in SEQ ID NO:6, and is exemplified by amino acids 410 (Lys410) through 442 (Leu442) (SEQ ID NO:18) (Figs. 1 and 3(a)). Preferably, a C-terminal portion of SEQ ID NO:6 includes at least about 6 contiguous amino acids from SEQ ID NO:18, more preferably at least about 14, even more preferably at least about 20. In a particularly preferred embodiment of this aspect of the invention, a C-terminal portion of SEQ ID NO:6 includes the last 14 amino acids of SEQ ID NO:6 (-Phe-Leu-Ser-Ile-Ser-Leu-Leu-Phe-Ile-Ser-Phe-Tyr-Leu-Leu, SEQ ID NO:13), which represents a novel hydrophobic signaling sequence for targeting to a ciliate membrane; even more preferably the C-terminal portion includes the last 23 amino acids of SEQ ID NO:6 (-Gln-Cys-Ala-Ser-Thr-Thr-Phe-Ala-Lys-Phe-Leu-Ser-Ile-Ser-Leu-Leu-Phe-Ile-Ser-Phe-Tyr-Leu-Leu, SEQ ID NO:14). Most preferably, the polynucleotide fragment of this aspect of the invention has a nucleotide sequence selected from the class of nucleotide sequences that encodes a polypeptide having SEQ ID NO:6.

It is envisioned that the C-terminal membrane targeting sequence of SEQ ID NO:6, exemplified by SEQ ID NOs:13 and 14, can be fused to other proteins and use to direct membrane surface display of the fused proteins in other ciliates known to utilize GPI anchors, such as *Tetrahymena* and *Paramecium*.

Conservative substitutions of hydrophobic residues, discussed in more detail below, such as substitutions of leucine, isoleucine, and phenylalanine with each other, are expected to be well tolerated within these signal sequences, and nucleotide sequence encoding SEQ ID NOs: 13 and 14 that have been modified with one or more of these conservative substitutions are also included in the invention.

One example of the class of nucleotide sequences that encodes a polypeptide having amino acid SEQ ID NO:6 is SEQ ID NO:1. This class of nucleotide sequences is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code, as used in *E. coli*. Additional members of this class of nucleotide sequences, for use in ciliate expression systems, can be determined using the modified genetic code for ciliates, as described herein.

Likewise, the invention includes a nucleic acid molecule comprising a polynucleotide fragment having a nucleotide sequence selected from the class of nucleotide sequences that encodes a polypeptide having at least a terminal portion of amino acid sequence SEQ ID NO:7. Preferably, the polynucleotide is at least about 20 nucleotides in length. A terminal portion of SEQ ID NO:7 can be an N-terminal portion or a C-terminal portion. An "N-terminal portion" of SEQ ID NO:7 includes at least one amino acid that is contiguous to, and located to the N-terminal side of, amino acid 21 (Ala21) in SEQ ID NO:7, and is exemplified by amino acids 1 (Met1) through 20 (Ser20) (SEQ ID NO:15). Preferably, an N-terminal portion of SEQ ID NO:7 includes at least about 6 contiguous amino acids from SEQ ID NO:15, more preferably at least about 10, even more preferably at least about 15. In a particularly preferred embodiment of this aspect of the invention, an N-terminal portion of SEQ ID NO:7 includes the first 20 amino acids of SEQ ID NO:7 (Met-Lys-Asn-Asn-Ile-Leu-Val-Ile-Leu-Ile-Ile-Ser-Leu-Phe-Ile-Asn-Gln-Ile-Lys-Ser, SEQ ID NO:15), which constitutes a novel membrane targeting sequence. A "C-terminal portion" of SEQ ID NO:7 includes at least one amino acid that is contiguous to, and located to the C-terminal side of amino acid 448 (Ile448) in SEQ ID NO:7, and is

exemplified by amino acids 449 (Gln449) through 468 (Leu468) (SEQ ID NO:17). Preferably, a C-terminal portion of SEQ ID NO:7 includes at least about 6 contiguous amino acids from SEQ ID NO:17, more preferably at least about 14, even more preferably at least about 20. In a particularly preferred embodiment of this aspect of the invention, a C-terminal portion of SEQ ID NO:7 includes the last 14 amino acids of SEQ ID NO:7 (Phe-Leu-Ser-Ile-Ser-Leu-Leu-Leu-Ile-Ser-Tyr-Tyr-Leu-Leu, SEQ ID NO:16), which represents a novel hydrophobic signaling sequence for targeting to a ciliate membrane; more preferably the C-terminal portion includes the last 20 amino acids of SEQ ID NO:7 (Gln-Cys-Asp-Phe-Ala-Asn-Phe-Leu-Ser-Ile-Ser-Leu-Leu-Leu-Ile-Ser-Tyr-Tyr-Leu-Leu, SEQ ID NO:17).

It is envisioned the N-terminal and C-terminal membrane targeting sequences of SEQ ID NO:7, exemplified by SEQ ID NOs:15-17, can be fused to other proteins and used to direct secretion or membrane surface display of the proteins in other ciliates known to utilize GPI anchors, such as *Tetrahymena* and *Paramecium*. Additionally, the N-terminal membrane targeting sequence may be more generally applicable to other protein expression systems. Conservative substitutions of hydrophobic residues, such as substitutions of leucine, isoleucine, and phenylalanine with each other, are expected to be well tolerated within these signal sequences, and nucleotide sequence encoding SEQ ID Nos:15-17 that have been modified with one or more of these conservative substitutions are also included in the invention.

Examples of the class of nucleotide sequences that encode a polypeptide having amino acid SEQ ID NO:7 are SEQ ID NOs:3 and 5. This class of nucleotide sequences is likewise large but finite, and the nucleotide sequence of each member of the class can also be readily determined by reference to the standard genetic code, as used in *E. coli*. Additional members of this class of nucleotide sequences, for use in ciliate expression systems, can be determined using the modified genetic code for ciliates, as described herein.

The nucleic acid molecule of the invention can be DNA, RNA, or a combination thereof, and can include any combination of naturally occurring,

chemically modified or enzymatically modified nucleotides. The nucleic acid molecule can be equivalent to the polynucleotide fragment encoding an i-antigen protein, or it can include said polynucleotide fragment in addition to one or more additional nucleotides or polynucleotides. For example, the nucleic acid molecule of the invention can be a vector, such as an expression or cloning vector. A vector useful in the present invention can be circular or linear, single-stranded or double stranded, and can include DNA, RNA, or any modification or combination thereof. The vector can be a plasmid, a cosmid, or a viral vector, such as baculovirus. Preferably, the nucleic acid molecule of the invention takes the form of an expression vector that is capable of expression in an organism or in a cell of the organism, in culture or in vivo. An organism or cell in which the coding sequence of the vector can be expressed can be eukaryotic or prokaryotic, and can be, without limitation, a bacterium, a yeast, an insect, a protozoan, preferably a ciliate such as *Tetrahymena*, or animal, such as a fish or a mammal. Preferably, the vector is expressible in a fish and/or in a conventional protein expression system, including bacteria, such as *E. coli*, yeast, such as *Pischia pastoris*, mammalian cell culture or insect cells.

When the vector is intended for use in bacterial, yeast, mammalian or insect expression systems, the coding sequences of the vector are preferably engineered to utilized the conventional genetic code rather than the ciliate genetic code that is employed in the native *I. multifiliis* coding sequences. Thus, in preferred embodiments of these aspects of the invention, the nucleotide sequence of the polynucleotide fragment that encodes an i-antigen protein is altered such that each ciliate glutamine codon TAA and TAG in the nucleotide sequence derived from the *I. multifiliis* isolate is replaced with a universal glutamine codons, either CAG or CAA. Accordingly, a particularly preferred embodiment of the nucleic acid molecule of the invention includes a polynucleotide fragment having SEQ ID NO:5.

It should be understood that the nucleic acid molecule of the invention can be single-stranded or double-stranded, and further that a single-stranded nucleic acid molecule of the invention includes a polynucleotide fragment

having a nucleotide sequence that is complementary to a nucleotide sequence that encodes an i-antigen protein or portion thereof according to the invention. As used herein, the term "complementary" refers to the ability of two single stranded polynucleotide fragments to base pair with each other, in which an adenine on one nucleic acid fragment will base pair to a thymine on the other, and a cytosine on one nucleic acid fragment will base pair to a guanine on the other. Two polynucleotide fragments are complementary to each other when a nucleotide sequence in one nucleic acid fragment can base pair with a nucleotide sequence in a second nucleic acid fragment. For instance, 5'-ATGC and 5'-GCAT are fully complementary, as are 5'-ATGC and 5'-GCAT.

Further, the single-stranded nucleic acid molecule of the invention also includes a polynucleotide fragment having a nucleotide sequence that is substantially complementary to a nucleotide sequence that encodes an i-antigen protein or portion thereof according to the invention, or to the complement of the nucleotide sequence that encodes an i-antigen or portion thereof. Substantially complementary polynucleotide fragments can include at least one base pair mismatch, such that at least one nucleotide present on a first polynucleotide fragment will not base pair to at least one nucleotide present on a second polynucleotide fragment, however the two polynucleotide fragments will still have the capacity to hybridize. For instance the middle nucleotide of each of the two DNA fragments 5'-AGCAAATAT and 5'-ATATATGCT will not base pair, but these two nucleic acid fragments are nonetheless substantially complementary as defined herein. Two polynucleotide fragments are substantially complementary if they hybridize under hybridization conditions exemplified by 2X SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate, pH 7.6) at 55°C. Substantially complementary polynucleotide fragments for purposes of the present invention preferably share at least one region of at least about 50 nucleotides in length, which shared region has at least about 85% nucleotide identity, preferably at least about 90% nucleotide identity. More preferably, substantially complementary polynucleotide fragments share a plurality of regions of between about 50 nucleotides and about 150 nucleotides in length,

which shared regions have at least about 85% nucleotide identity, preferably at least about 90% nucleotide identity. In a particularly preferred embodiment, the substantially complementary nucleotide sequence encodes at least one of SEQ ID NOs:90-100 as shown on Fig. 3(a). Locations and levels of nucleotide sequence identity between two nucleotide sequences can be readily determined using CLUSTALW multiple sequence alignment software.

The invention further includes a nucleic acid molecule comprising a polynucleotide fragment that hybridizes to at least a portion of the complement of either or both of SEQ ID NO:1 or SEQ ID NO:3, under standard hybridization conditions, provided that the polynucleotide fragment encodes a polypeptide comprising at least a membrane targeting portion or an antigenic portion of an i-antigen protein. A membrane targeting portion of an i-antigen protein is one that targets the polypeptide to either the endoplasmic recticulum (e.g., an N-terminal signal sequence) or to the plasma membrane (e.g., a GPI anchor sequence). Standard hybridization conditions are exemplified by 2X SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate, pH 7.6) at 55°C.

The invention further includes a nucleotide molecule comprising a polynucleotide fragment encoding an antigenic analog or modification of a polypeptide represented by SEQ ID NO:6, or an antigenic fragment thereof that includes at least a C-terminal portion of SEQ ID NO:6; further, the invention includes a nucleotide molecule comprising a polynucleotide fragment encoding an antigenic analog, fragment, or modification of a polypeptide represented by SEQ ID NO:7, as described in more detail below.

Also provided by the invention is a novel i-antigen protein. In a further aspect of the invention, therefore, the i-antigen protein is encoded by a nucleotide sequence derived from *I. multifiliis* (G1 isolate), and has a molecular weight of about 48 kD. In a particularly preferred embodiment of this aspect of the invention, the i-antigen protein is encoded by the nucleotide sequence as shown in Fig. 3(b), nucleotides 1 through 1326 (SEQ ID NO:1), representing the coding region of the *IAG48[G1]* gene of *I. multifiliis*, and has the amino acid sequence SEQ ID NO:6 (Fig. 1). In another aspect of the invention, the i-

antigen protein is encoded by a nucleotide sequence derived from *I. multifiliis* (G5 isolate), and has a molecular weight of about 55 kD. In a particularly preferred embodiment of this aspect of the invention, the i-antigen is encoded by the nucleotide sequence as shown in Fig. 2(a), nucleotides 1 through 1404 (SEQ ID NO:3), representing the coding region of the *IAG55[G5]* gene, and has the amino acid sequence SEQ ID NO:7 (Fig. 4).

The i-antigen polypeptide of the invention includes an i-antigen polypeptide having SEQ ID NO:6; an i-antigen polypeptide having SEQ ID NO:7; an analog or modification of an i-antigen polypeptide having SEQ ID NO:6; a fragment of an i-antigen polypeptide having SEQ ID NO:6 having at least a C-terminal portion of SEQ ID NO:6; an antigenic analog, fragment, or modification of an i-antigen polypeptide having SEQ ID NO:7; and an analog, fragment, or modification of an i-antigen polypeptide having SEQ ID NO:7 wherein said analog, fragment or modification has at least one terminal portion of SEQ ID NO:7.

An antigenic analog, fragment, or modification of a polypeptide having SEQ ID NOs:6 or 7 is one that generates an immune response in fish against *I. multifiliis*. Antigenicity of an polypeptide can be evaluated *in vitro* by performing a Western blot on the purified polypeptide (for example, an affinity purified polypeptide) using polyclonal antisera from a rabbit that was vaccinated with at least an antigenic portion of a native *I. multifiliis* i-antigen protein, preferably with a complete *I. multifiliis* i-antigen protein (e.g., SEQ ID NO:6 or SEQ ID NO:7).

Antigenic analogs of polypeptide having SEQ ID NO:6, SEQ ID NO:7 include i-antigen polypeptides having amino acid substitutions that do not eliminate polypeptide antigenicity in fish. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine

and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH_2. Fragments of an i-antigen polypeptide of the invention include i-antigen polypeptides containing deletions or additions of one or more contiguous or noncontiguous amino acids that do not eliminate the antigenicity of the i-antigen in fish are also contemplated. Fragments of an i-antigen polypeptide contain at least about six amino acids, preferably at least about 10 amino acids, more preferably at least about 60 amino acids. Modified i-antigens include i-antigens that are chemically and enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N– and C– terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

The invention further includes an antigenic polypeptide that shares a significant level of primary structure with either or both of SEQ ID NO:6 or SEQ ID NO:7. Preferably, the antigenic polypeptide of this aspect of the invention is a synthetic polypeptide. A synthetic polypeptide is one that does not have the amino acid sequence of a polypeptide that is isolated from an organism; i.e., it is not a naturally occurring polypeptide. An antigenic polypeptide shares a significant level of primary structure with either or both of SEQ ID NO:6 or SEQ ID NO:7 if it has a plurality of amino acid sequence domains, each domain having about 60 to about 100 amino acids, of which six are cysteines that fall into register when the sequence domains are aligned with the amino acid sequence domains of SEQ ID NOs:6 or 7, as exemplified in Fig. 5. The predominant primary structure motif in an antigenic polypeptide of the invention is -Cys-$Xaa_{2,3}$-Cys- (SEQ ID NOs:31, 32), where Xaa is any amino acid, and where $Xaa_{2,3}$ means Xaa_2 or Xaa_3, that is, where pairs of cysteines are separated by two or three amino acids. Preferably, the antigenic polypeptide of the invention has larger scale repeating motifs characterized by -Cys-Xaa_2-Cys-

Xaa_m-Cys-Xaa_3-Cys-Xaa_n-Cys-Xaa_2-Cys- where m = 15 - 25, preferably 20-22, and n = 15-25, preferably 19-20 (e.g., SEQ ID NO:33 where m = 20, n = 20). More preferably, the larger scale repeating motifs are characterized by -Cys-Xaa_2-Cys-Xaa_m-Cys-Xaa_3-Cys-Pro-Xaa_p-Cys-Xaa_2-Cys- where m = 15-25, preferably 20-22 and p = 14-24, preferably 18-19 (e.g., SEQ ID NO:88 where m = 20, p = 18). In a particularly preferred embodiment, the larger scale repeating motifs are characterized by -Cys-Xaa_2-Cys-Xaa_q-Gln-Cys-Xaa_3-Cys-Pro-Xaa-Gly-Thr-Xaa_r-Cys-Xaa_2-Cys-, where q = 14-24, preferably 19-21, and r = 11-21, preferably 15-16 (e.g., SEQ ID NO:89 where q = 20, r = 20). In a particularly preferred embodiment of the invention, each amino acid sequence domain of an antigenic polypeptide of the invention has at least 90% amino acid identity with at least one of SEQ ID NOs:8-12 (Fig. 5(a)) and 55-60 (Fig. 5(b)); most preferably, each amino acid sequence domain of an antigenic polypeptide of the invention has at least 95% amino acid identity with at least one of SEQ ID NOs:8-12 and 55-60 (Fig. 5).

The location and level of amino acid sequence identity between two amino acid sequences can be readily determined using CLUSTALW multiple sequence alignment software. Alternatively, the two amino acid sequences (i.e., the amino acid sequence of the candidate domain sequence of the antigenic polypeptide and the reference amino acid sequence selected from SEQ ID NOs:8-12 and 55-60) are aligned such that the cysteines are in register, then further aligned to maximize the number of amino acids that they have in common along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to place the cysteines in register and to maximize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The percentage amino acid identity is the higher of the following two numbers: (a) the number of amino acids that the two sequences have in common within the alignment, divided by the number of amino acids in the reference sequence, multiplied by 100; or (b) the number of amino acids that the two sequences have in common

within the alignment, divided by the number of amino acids in the candidate polypeptide, multiplied by 100.

The invention further includes a polypeptide having a membrane targeting sequence selected from a C-terminal portion of SEQ ID NO:6, an N-terminal portion of SEQ ID NO:7, and a C-terminal portion of SEQ ID NO:7, as described above.

The present invention further provides a vaccine for use in preventing or controlling disease in fish caused by *I. multifiliis*. The polynucleotide vaccine comprises a polynucleotide fragment, preferably a DNA fragment, having a nucleotide sequence encoding an antigenic polypeptide comprising at least an antigenic portion of an i-antigen protein derived from *I. multifiliis*. The polynucleotide vaccine optionally further comprises a promoter, preferably the CMV promoter, operably linked to the coding sequence for the i-antigen polypeptide (e.g., ~~U.S. Pat. No. 5,708,448~~ U.S. Pat no. 5,780,448, Davis). There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccines. A specific embodiment employs constructs using the plasmid pcDNA3.1 as the vector (InVitrogen Corporation, Carlsbad, CA). In addition, the vector construct can contain immunostimulatory sequences (ISS), such as unmethylated dCpG motifs, that stimulate the animal's immune system. Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences encoding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12) and co-stimulatory molecules such B7-1, B7-2, CD40. The cytokines can be used in various combinations to fine-tune the response of the animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to affect the animal's reproductive system. The polynucleotide vaccine can also encode a fusion product containing the antigenic polypeptide and a molecule, such as CTLA-4, that directs the fusion product to antigen-presenting cells inside the host. Plasmid DNA can also be delivered using attenuated bacteria as delivery system, a method that is suitable for DNA vaccines that are administered orally. Bacteria are transformed with an

independently replicating plasmid, which becomes released into the host cell cytoplasm following the death of the attenuated bacterium in the host cell. An alternative approach to delivering the polynucleotide to an animal involves the use of a viral or bacterial vector. Examples of suitable viral vectors include adenovirus, polio virus, pox viruses such as vaccinia, canary pox, and fowl pox, herpes viruses, including catfish herpes virus, adenovirus-associated vector, and retroviruses. Exemplary bacterial vectors include attenuated forms of *Salmonella*, *Shigella*, *Edwardsiella ictaluri*, and *Yersinia ruckerii*. Preferably, the polynucleotide is a vector, such as a plasmid, that is capable of autologous expression of the nucleotide sequence encoding an i-antigen.

In a particularly preferred embodiment, the vaccine is a DNA vaccine comprising a DNA fragment having a nucleotide sequence that encodes a polypeptide having amino acid sequence SEQ ID NO:6 or SEQ ID NO:7, an antigenic analog, fragment, or modification of a polypeptide having SEQ ID NO:7, an antigenic analog or modification of a polypeptide having SEQ ID NO:6, or an antigenic fragment of a polypeptide having SEQ ID NO:6 provided that the fragment includes at least a C-terminal portion of SEQ ID NO:6. An antigenic analog, fragment, or modification of an i-antigen polypeptide is one that generates an immune response in fish against *I. multifiliis*. For example, a preferred DNA vaccine comprises a DNA fragment having a nucleotide sequence encoding an amino acid sequence selected from the group consisting of SEQ ID NOs:8-12 and 55-60; these eleven amino acid sequences represent tandemly repeated amino acid sequence domains found in the deduced amino acid sequence SEQ ID NO:6 and are expected to be antigenic. More preferably, the DNA vaccine comprises all or a portion of nucleotide sequence SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, provided that the portion of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 encodes an antigenic polypeptide and further provided that, in SEQ ID NO:1 and SEQ ID NO:3 codons TAA and TAG that code for glutamine in *I. multifiliis* are changed to CAG or CAA. Most preferably, the DNA vaccine includes a synthetic DNA fragment having a

nucleotide sequence that encodes a polypeptide SEQ ID NOs:6 or 7 using codons that are biased in favor of the host's codon usage.

Polynucleotide-based immunization induces an immune response to an antigen expressed *in vivo* from a heterologous polynucleotide fragment introduced into the fish. This method can be advantageous over other methods because heterologous nucleic acid expression may continue for a length of time sufficient to induce a relatively strong and sustained immune response without the need for subsequent "booster" vaccinations, as is common when portions of the protein antigen itself have been injected into the animal. A polynucleotide vaccine comprising a polynucleotide fragment having a nucleotide sequence encoding an i-antigen protein can be administered to a fish using biolistic bombardment, bath immersion, ingestion or direct injection, as described in U.S. Pat. No. 5,780,448 (Davis), preferably intraperitoneal or intramuscular injection. A preferred method of administration is biolistic bombardment, as with a "gene gun." A polynucleotide vaccine formulated for oral administration preferably contains DNA encapsulated in a biodegradable polymer. Examples of a suitable biodegradable polymer include chitosan and homo- or co-polymers of polylactic acid and polyglycolic acid. The invention thus further provides a method for immunizing freshwater fish against *I. multifiliis* by administering to the fish a polynucleotide vaccine of the invention, preferably a DNA vaccine.

The amount of polynucleotide vaccine to be administered to an animal depends on the type and size of animal, the condition being treated, and the nature of the polynucleotide, and can be readily determined by one of skill in the art. In fish, for example, if the polynucleotide vaccine is to be injected, the amount per injection is preferably at least about 10 ng; at most it is preferably about 50 μg, more preferably it is less than about 1 μg. If the polynucleotide vaccine is to be administered using a gene gun, the amount per dose is preferably at least about 1 ng; at most it is preferably about 10 μg, more preferably it is less than about 1 μg. For administration by immersion, the concentration of the polynucleotide in the aquatic medium is preferably at least about 10 ng/mL; at most it is preferably about 50 μg/mL, preferably it is less than about 1 μg/mL.

For oral administration the amount per dose is preferably at least about 10 ng; at most it is preferably about 10 μg, preferably less than about 1 μg. In some applications, one or more booster administrations of the vaccine at time periods subsequent to the initial administration are useful to create a higher level of immune response in the animal.

In another aspect, the vaccine of the invention comprises an i-antigen polypeptide having amino acid sequence SEQ ID NO:6 or SEQ ID NO:7, an antigenic analog, fragment, or modification of a polypeptide having SEQ ID NO:7, an antigenic analog or modification of a polypeptide having SEQ ID NO:6, or an antigenic fragment of a polypeptide having SEQ ID NO:6 provided that the fragment includes at least a C-terminal portion of SEQ ID NO:6. This type of vaccine is referred to herein as a "protein subunit vaccine" even if it contains the entire i-antigen sequence. The i-antigen or antigenic analog, fragment, or modification thereof for use in the protein subunit vaccine of the invention can be naturally occurring (i.e., isolated from *I. multifiliis*) or recombinant. A protein subunit vaccine of the invention are conveniently administered to fish using bath immersion, ingestion, topical administration, or direct injection, preferably intraperitoneal or intramuscular injection. A protein subunit vaccine formulated for oral administration preferably contains polypeptide encapsulated in a biodegradable polymer as described above in connection with the polynucleotide vaccine of the invention. In addition, the protein subunit vaccine can be administered to an animal via a live vector, such as recombinant *Tetrahymena*. *Tetrahymena* can be transformed such that it expresses the i-antigen or antigenic analog, fragment, or modification thereof, either in the cytosol, as a transmembrane protein, as a GPI-anchored protein or as a secreted protein. Recombinant *Tetrahymena* can be injected into the animal or, in the case of an aquatic animal such as a fish, can be administered via immersion. Oral administration of *Tetrahymena* if also envisioned. The invention thus further provides a method for immunizing freshwater fish against *I. multifiliis* by administering to the fish a protein subunit vaccine of the invention.

The amount of protein subunit vaccine to be administered to an animal depends on the type and size of animal, the condition being treated, and the nature of the protein, and can be readily determined by one of skill in the art. In fish, for example, if the protein subunit vaccine is to be injected, the amount per injection is preferably between about 0.1 μg and about 1000 μg per 10g fish; more preferably it is between about 1 μg and about 100 μg per 10 g of fish. For administration by immersion, the concentration of the protein in the aquatic medium is preferably at least about 10 ng/mL; at most it is preferably about 50 μg/mL, preferably it is less than about 1 μg/mL. For oral administration the amount per dose is preferably between about 0.1 μg and about 100 μg per 10g fish; more preferably it is between about 1 μg and about 10 μg per 10 g of fish. Preferably, the protein subunit vaccine also includes an adjuvant. Further, one or more boosters are preferably administered at time periods subsequent to the initial administration to create a higher level of immune response in the animal.

In yet another aspect, the vaccine of the invention comprises a fusion protein comprising a carrier polypeptide and an i-antigen polypeptide of the invention or an analog, fragment, or modification thereof. An i-antigen analog, fragment, or modified i-antigen for use in this aspect of the invention can itself be antigenic or nonantigenic; in embodiments wherein the i-antigen analog, fragment or modified i-antigen is nonantigenic, the carrier polypeptide provides the necessary antigenicity by stimulating the fish's immune system to react to the fusion protein thereby generating an immune response in fish against *I. multifiliis*. A nonantigenic analog, fragment, or modification of the i-antigen thus function as a hapten. An example of an antigenic carrier polypeptide is KLH. Conventional fusion constructs between carriers such as glutathione sulfotransferase (GST) and i-antigens of the invention or antigenic analog, fragment, or modifications thereof are also included as protein subunit vaccines according to the invention, as are fusions of the i-antigen and an affinity tag such as a polyhistidine sequence. A fusion construct may be preferred for use as a protein subunit vaccine when the antigenic i-antigen analog, fragment, or modification thereof is small. The invention further provides a method for

immunizing freshwater fish against *I. multifiliis* by administering to the fish a fusion protein vaccine of the invention.

Monoclonal antibodies that recognize immobilizing epitopes on i-antigens are protective in passive immunization experiments, but their activity is serotype-specific. On the other hand, fish that are actively immune following exposure to one serotype are cross-protected against heterologous strains. Thus, in one embodiment, the vaccine of the invention (whether in the form of a protein vaccine or a polynucleotide vaccine) is monovalent in that it is derived from a particular i-antigen from a particular serotype of *I. multifiliis* and effective to treat or prevent infection of the vaccinated animal by that serotype. Preferably, the monovalent vaccine contains at least one antigenic determinant that is shared by i-antigens of different serotypes, such that it also prevents infection by other *I. multifiliis* of other serotypes, thus offering broad protection. In another embodiment, the vaccine of the invention (whether in the form of a protein vaccine or a polynucleotide vaccine) is a combined vaccine or a multivalent vaccine that prevents infection by other *I. multifiliis* of more than one serotype. The combined or multivalent vaccine can contain or encode, for example, a plurality of serotype-specific i-antigen polypeptides or antigenic portions thereof, derived from multiple serotypes of *I. multifiliis*, or can contain or encode a synthetic or fusion i-antigen polypeptide containing multiple antigenic determinants that together generate an immune response against multiple serotypes of *I. multifiliis*.

In a preferred embodiment of the vaccine of the invention, i-antigen or an antigenic portion thereof is linked at its carboxy-terminus to at least two molecules of the C3d component of complement, using molecular cloning techniques. Preferably, the i-antigen or antigenic portion thereof is linked to about three molecules of the C3d component of complement. The C3d molecule can be either homologous or heterologous with respect to the species to be vaccinated. Complement genes have been cloned and characterized in salmonids (J. Lambris et al., J. Immunol. 151:6123 (1993); J. Sunyer et al., Proc. Natl. Acad. Sci USA 93:8546 (1996)). For vaccinations of fish, the i-antigen or

antigenic portion thereof is preferably linked to a salmonid C3d, such as trout C3d or catfish C3d. In the case of a protein subunit vaccine, the recombinant protein is conveniently expressed in bacteria, then administered to fish. This technique has been shown to generate in increase in the immune response in mice (P. Dempsey et al., Science ~~271:48~~ 271: 348-350 (1996)). The receptor for C3d, namely CD21, is expressed primarily on B cells and the follicular dendritic cells of lymphoid tissues. In the case of a polynucleotide vaccine, a plasmid encoding a fusion protein that incorporates an i-antigen or antigenic portion thereof, linked at its carboxy-terminus to at least two molecules of the C3d component, is administered to the fish.

The active immune-stimulating ingredients are optionally mixed with excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the active ingredient. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the immune-stimulating composition (including vaccine) may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition.

Fish that can be vaccinated against Ich infection include freshwater teleosts, preferably fish that are widely farmed in aquaculture including channel catfish, trout, salmon, tilapia and eels. The goal of vaccination against Ich infection is to elicit a population of lymphocytes, which upon subsequent exposure to the parasite proliferate and produce antibodies and/or effector cells specific to the parasite, resulting in protection against lethal infections. A vaccine effective for the prevention of Ich infection in freshwater fish is thus one which elicits the production of protective antibodies in a fish exposed to said vaccine. Those protective antibodies will prevent lethal infection of the vaccinated fish upon challenge with *I. multifiliis*. In the present invention,

protective antibodies generated in the fish are specific for *I. multifiliis* i-antigens. Fish that can be immunized include ornamental and food fish.

The present invention further includes monoclonal or polyclonal antibodies, whether derived from fish, rodents, mammals, avians, or other organisms, that bind to the i-antigen proteins described herein, including antigenic analogs, fragments and modifications thereof. Production and isolation of monoclonal and polyclonal antibodies to a selected polypeptide sequence is routine in the art.

Sera from immune fish is known to confer passive immunity against both viral and bacterial pathogens when injected into non-immune fish (Hedrick et al. Trans. Amer. Fish Soc. 116:277 (1987); Viele et al. J. Fish Biol. 17:379 (1980)). We have carried out similar experiments in which mouse monoclonal antibodies specific for Ich i-antigen(s) of the A and D serotypes were transferred into non-immune 10-15 g catfish fingerlings. Monoclonal antibodies which have the ability to immobilize these serotypes *in vitro* conferred passive protection against the parasite *in vivo*. Fish weighing 10-15 g were injected intraperitoneally with 20 -200 μg of purified mouse monoclonal antibody (MAb) and challenged with infective theronts after 24 hours. A serotype (G1-specific) MAbs included 10H3, 3H12, 8E11, 6A11 and 5A8; D serotype (G5 and G3-specific) MAbs included G3-27 and G3-61. Animals injected with immobilizing MAbs survived lethal infection while controls were completely overwhelmed. The passive protection achieved in the channel catfish model supports the use of i-antigen protein(s) as vaccines for eliciting active immunity (Lin et al., Inf. Immun. 4085-4090 (1996); see also He et al. (Aquaculture 158:1-10 (1997)).

Comparison of newly discovered amino acid sequences SEQ ID NO:6 (the G1 *I. multifiliis* isolate) and SEQ ID NO:7 (the G5 *I. multifiliis* isolate) shows regions of amino acid identity that were heretofore unknown and unpredictable, because the sequence of only one *I. multifiliis* i-antigen (i.e., the sequence encoded by the cDNA for the 48 kD protein, Clark et al. (Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992) was known. Fig. 3(a) shows regions of identity or high homology, which are boxed. It is expected that an

oligonucleotide probe or primer having a sequence that encodes a conserved region of the i-antigens as identified herein will be useful for identifying additional i-antigens in *I. multifiliis* having other serotypes; molecular methods for constructing these probes and primers and using them to screen genomic libraries are routine in the art. For example, amino acid sequences at the N and C- termini are highly conserved (see Fig. 3(a)). The corresponding regions of the genes would likely serve as sites for the design of diagnostic PCR primers that could be used to amplify i-antigen sequences from any parasite isolate. Moreover, using mouse monoclonal antibodies against the i-antigens, we have been able to define differences between parasite isolates, and have shown that protection following passive antibody transfer is strain (i.e., isolate) specific. Thus, the identification of additional i-antigens using nucleotide probes and primers derived from conserved regions as described herein will likely provide amino acid and nucleotide sequence information that allows the production of additional serotype-specific i-antigen DNA and protein subunit vaccines.

Accordingly, the invention further provides for an oligonucleotide probe or primer (including its complement) having a nucleotide sequence encoding at least a portion of an i-antigen conserved region as shown in Fig. 3(a) (boxed regions). Preferably, the oligonucleotide probes or primers are represented by nucleotide sequences that encode conserved *I. multifiliis* i-antigen regions of at least about eight amino acids in length, which conserved regions are selected from portions of SEQ ID NOs:90-100 (Fig. 3(a)) that contain at least about eight amino acids. More preferably, the oligonucleotide probes or primers are represented by the sequences MKYNILLT (SEQ ID NO:36), FLSISLLF (SEQ ID NO:38), GTALDDGV (SEQ ID NO:46), AGTDTCT (SEQ ID NO:48), CTKKLTSGA (SEQ ID NO:50) and FAKFLSISL (SEQ ID NO:52). Further, the invention provides a method for identifying i-antigens in *I. multifiliis* by using the oligonucleotide probe or primer of the invention to identify and isolate novel nucleotide sequences encoding other i-antigens, for example by probing a genomic DNA or cDNA library of an isolate of *I. multifiliis* or by conducting polymerase chain reaction. Vaccines that utilize the nucleotide and amino acid

sequences of the i-antigens so discovered, which as a result are effective against other serotypic variants of *I. multifiliis*, are also provided.

Knowledge of the i-antigen nucleotide and amino acid sequences set forth herein also opens up new possibilities for detecting, diagnosing and characterizing *Ichthyophthirius* in fish populations. For example, an oligonucleotide probe or primer based on a conserved region of the i-antigen protein can be used to detect the presence of *Ichthyophthirius* in a fish or in water, and an oligonucleotide probe or primer based on a less conserved region can be used to identify a specific *Ichthyophthirius* serotype. The invention therefore includes methods for detecting and characterizing *Ichthyophthirius*, for example in aquaculture facilities.

Recombinant Expression of I-antigens

As already noted, the principal difficulty associated with making a vaccine against *I. multifiliis* is the fact that this obligate parasite cannot be easily cultured. Thus, production of i-antigen in an recombinant system is highly desirable. Ciliated protozoans, however, including *Ichthyophthirius*, utilize TAA and TAG as codons for the amino acid glutamine, while most other organisms recognize those as termination codons. Therefore, according to the invention, either the native nucleotide sequence is expressed in a ciliated protozoan, or the native nucleotide sequence is altered to use a universal glutamine codon (either CAG or CAA) in place of any TAA and TAG triplets used in the native sequence.

Bacterial expression systems. Selection of a vector or plasmid backbone depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, plasmid reproduction rate, and the like. Suitable plasmids for expression in *E. coli*, for example, include pUC(X), pKK223-3, pKK233-2, pTrc99A, and pET-(X) wherein (X) denotes a vector family in which numerous constructs are available. pUC(X) vectors can be obtained from Pharmacia Biotech (Piscataway, NH) or Sigma Chemical Co. (St. Louis, MO). pKK223-3, pKK233-2 and pTrc99A can be obtained from Pharmacia Biotech. pET-(X)

vectors can be obtained from Promega (Madison, WI) Stratagene (La Jolla, CA) and Novagen (Madison, WI). To facilitate replication inside a host cell, the vector preferably includes an origin of replication (known as an "ori") or replicon. For example, ColE1 and P15A replicons are commonly used in plasmids that are to be propagated in *E. coli.*

The expression vector optionally includes a promoter sequence operably linked to the nucleotide sequence encoding i-antigen protein. A promoter is a DNA fragment which causes transcription of genetic material. Transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. The invention is not limited by the use of any particular promoter, and a wide variety are known. Promoters act as regulatory signals that bind RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence. A promoter is "operably linked" to a nucleic acid sequence if it is does, or can be used to, control or regulate transcription of that nucleic acid sequence. The promoter used in the invention can be a constitutive or an inducible promoter. It can be, but need not be, heterologous with respect to the host cell. Preferred promoters for bacterial transformation include *lac*, *lac*UV5, *tac*, *trc*, T7, SP6 and *ara*.

The nucleotide sequence encoding the i-antigen protein can advantageously be fused, at either the 5' or 3' end, to a nucleotide sequence encoding an affinity tag, such as a polyhistidine amino acid sequence. The resulting fusion construct can be conveniently purified using the affinity tag for subsequent use. Affinity tags and methods for protein purification using affinity tags are well-known in the art. Optionally, a cleavage site, such as a Factor X cleavage site, can be introduced between the affinity tag and the amino acid sequence of the i-antigen to facilitate large-scale preparation of the i-antigen protein free of the carrier polypeptide.

The expression vector optionally includes a Shine Dalgarno site (i.e., a ribosome binding site), and a start site (e.g., the codon ATG) to initiate translation of the transcribed message to produce the enzyme. It can also include a termination sequence to end translation. A termination sequence is typically a

codon for which there exists no corresponding aminoacetyl-tRNA, thus ending polypeptide synthesis. The nucleic acid fragment used to transform the host cell can optionally further include a transcription termination sequence. The rrnB terminators, which is a stretch of DNA that contains two terminators, T1 and T2, is the most commonly used terminator that is incorporated into bacterial expression systems (J. Brosius et al., J. Mol. Biol. 148:107-127 (1981)).

The TAA and TAG codons in the native coding sequence of the i-antigen can be substituted with conventional glutamine codons either by site-directed mutagenesis (see e.g., Sambrook et al. (1989) Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory Press, Plainview, NY) or by creating a synthetic coding sequence using chemical synthesis of the desired coding sequence. Manual DNA synthetic techniques are well known (see, e.g., Caruthers (1983) in ~~Methodology~~ Methods of DNA and RNA Sequencing, Weissman (ed.), Praeger Publishers, New York, Chapter 1), as is automated DNA synthesis using any of several commercially available systems.

An alternative strategy is to express the i-antigen coding sequence with the UAA and UAG glutamine codons in a suppressor strain of *E. coli*. Cohen et al. (J. Molec. Biol. 216:189-194 (1990)) has reported a plasmid vector (pAD205) which provides an inducible suppressor tRNA which recognizes UAA and UAG codons and which is charged with glutamic acid. The plasmid pAD205 contains the gene encoding the artificial suppressor $tRNA^{glu}$ su oc205, previously described by Raftery et al. (EMBO J. 6:1499-1506 (1987)) which gene is expressed in pAD205 under the regulatory control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter. IPTG induces the expression of the tRNAs and thus allows the readthrough of UAG and UAA stop codons. Basal levels of the suppressor tRNAs are kept low by the presence of a leaky transcription termination signal between the promoter and the tRNA gene to minimize potentially lethal effects on the cells. J. Cohen et al. (J. Mol. Biol. 216:189 (1990)) reported the expression of a *Paramecium* tubulin gene in *E. coli* using this vector.

Eukaryotic expression systems. The insect baculovirus vector, *Autographa californica*, is capable of high level expression of foreign gene inserts and posttranslational modifications (see, e.g., International Patent Publication WO 90/14428). In terms of qualitative considerations, posttranslational modifications, including glycosylation, may be crucial in stimulating appropriate antigenic responses in vaccinated animals. In this regard yeast vectors can be useful since both glycosylation and secretion of foreign proteins are possible (Innis et al., Science 228:21 (1985)). A commercially available system (Immunex Corp., Seattle, WA) which is advantageous from both qualitative and quantitative standpoints has recently been described (Hopp et al., Biotechnology 6:1204 (1988)). This system allows both the detection and simplified purification of fusion proteins from yeast supernatants or *E. coli* extracts.

Protozoan expression systems. *Tetrahymena* is a ciliated protozoan which is taxonomically related to *I. multifiliis*. *T. thermophila* recognizes UAA and UAG codons as glutamine codons as does *I. multifiliis*. In addition, posttranslational modifications, particularly glycosylation, are expected to occur more normally in organisms related to *Ichthyophthirius* (for example, *Tetrahymena*) than in procaryotes such as *E. coli*, or more distantly related eucaryotes. Because such modifications can play a critical role in immune recognition, *Tetrahymena* can have an advantage on this level as well. Vectors for and gene expression in *Tetrahymena thermophila* have been reported. For example, *T. thermophila* has been successfully transformed using self-replicating palindromic ribosomal DNA (rDNA) purified from macronuclei (Brunk et al., Exp. Cell Res. ~~162:390 (1988))~~ 162:390-400 (1986), Lovlie et al., Proc. Natl. Acad. Sci. USA 85:~~5156~~ 5160 (1988); Tondravi et al., Proc. Natl. Acad. Sci. USA 83:4369 4373 (1986)). A selectable paromomycin resistance marker has been isolated and characterized; the resistant phenotype is due to a point mutation in the 17S rRNA gene. Resistance to hydromycin is conferred by this mutation as well (~~Sprangler~~ Spangler et al., J. Biol. Chem. 260:6334 6340 (1985)). Subsequently shuttle vectors capable of autonomously replicating as plasmids in *Tetrahymena* as well as in *E.*

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coli have been developed (Yu et al., Proc. Natl. Acad. Sci USA 86:8487 (1989)); Yu et al., Proc. Natl. Acad. Sci. USA 85:5151 (1988)). Such plasmids have been stably maintained at high copy number for more than 65 generations, and there has been at least one homologous gene expressed from a *Tetrahymena* shuttle vector.

Successful expression of an Ich gene in *T. thermophila* allows the production of relatively large amounts of antigen in a purified form, at relatively low cost. In addition to its use in the production of purified antigens, transformed *T. thermophila* can also be used as a live vector in the animal host, as described above. This second approach provides the most efficient means of exposing fish to *I. multifiliis* surface antigens (short of infecting them with the live parasite itself). Up to 500,000 *T. thermophila* cells can be injected into the peritoneal cavity of 15 g channel catfish, where they survive for several days without causing adverse effects.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

Isolation and Characterization of the *I. multifiliis* (G1 Isolate) Gene Encoding the 48 kD I-antigen

Parasite culture. *Ichthyophthirius multifiliis* (isolate G1) was maintained on juvenile channel catfish (*Ictalurus punctatus*) as previously described (H. Dickerson et al., J. Euk. Microbiol., 40:816-820(1993)). Briefly, the infection is passsaged on fingerling channel catfish by incubation of one infected fish with five uninfected fish in a 10 gallon aquarium. Fish were gently rubbed and host-

associated trophonts that were released from the skin were allowed to adhere to the sides of collecting dishes. Cells were then washed once in carbon-filtered tap water and permitted to divide to form infective theronts (18-24 hours at 23°C). After filtration through 0.0015 inch wire mesh (Fisher Scientific, Pittsburgh, PA) cells were used for DNA isolation.

Genomic DNA isolation. Total DNA was obtained using a modification of procedures described by Kavenoff et al. (Chromosoma, 41:1-27 (1973)). Theronts were harvested by centrifugation at 1,000 X g for 2 minutes and the resulting cell pellet lysed in 2 volumes HET buffer (0.5 M EDTA, 0.01 M Tris·HCl, pH 9.5) containing 1% SDS at 65°C. After 30 minutes, samples were cooled to 50°C and additional HET containing pronase (2 mg/ml) was added to bring the final volume to 4 times that of the original cell pellet. Lysates were incubated 4-6 hours, diluted with 0.5 vol 0.1 X SSC (0.1X SSC: 15 mM NaCl/1.5 mM sodium citrate, pH 7.0) and then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After separation from the aqueous layer, the organic phase was back-extracted with 0.5 volume of 10 mM Tris·HCl, 1 mM EDTA, pH 8.0 (TE) and the aqueous layers combined. Samples were re-extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), and nucleic acids were precipitated by addition of 2 volumes ethanol. The precipitate was collected by centrifugation at 10,000 x g for 15 minutes (4°C), washed in cold 70% ethanol and air dried. The pellet was dissolved in 2 X SSC containing 1 mM EDTA, and contaminating RNA was removed by digestion with 100 µg/ml RNase A (Promega) at 37°C for 1 hour. DNA was precipitated by addition of 2 volumes ethanol and collected by centrifugation as above. The pellet was washed for several hours in cold 70% ethanol and the DNA dissolved in TE to a final concentration of 50-250 µg/ml.

Genomic library construction. Total DNA (20 µg) was digested overnight at 37°C with the restriction endonuclease *Swa* I (4.2 units enzyme/µg DNA) (Boehringer-Mannheim Biochemicals, Indianapolis, IN). This enzyme has an 8 base pair recognition sequence containing all As and Ts. Because the

non-coding regions of ciliate DNA are extremely A-T rich, *Swa* I cuts preferentially and relatively often in intergenic regions yielding roughly gene-sized pieces of DNA. After phenol extraction, restriction fragments were ethanol precipitated and dissolved in TE (10 mM Tris/1 mM EDTA pH 8.0. Potential staggered ends were repaired by incubation with T4 DNA polymerase (0.2 unit/μl for 30 minutes at 37°C) (Promega, Madison, WI), and small fragments (<271 base pairs (bp)) removed by chromatography on Sephacryl 400 (Amersham Pharmacia Biochemical, Piscataway NJ). Genomic DNA (average size ~ 1.5-3 kb) was combined with a 20-fold molar excess of 10_{mer} *Eco*R I adaptors (e.g., Cat. No. C-1291, Promega) and the mixture incubated for 18 hours at 15°C in the presence of T4 DNA ligase (0.25 unit/μl) (Promega). T4 polynucleotide kinase (to 0.25 unit/μl; Promega) and ATP (to 10 nM) were added and the reactions incubated an additional 30 minutes at 37°C to phosphorylate adaptor ends. Fragments were phenol extracted and ethanol precipitated as above, and again fractionated on Sephacryl 400 to remove excess adaptors. Resulting DNA was then ligated with λZAP II arms (Stratagene, La Jolla, CA) which had been *Eco*R I digested and dephosphorylated. Ratios of insert to vector DNA were varied according to the recommendations of the manufacturer. Individual ligation reactions were packaged using Gigapack II packaging extracts (Stratagene) and libraries titered on *E. coli* XL1-Blue MRF' in the presence of X-gal and IPTG. A library containing 4 X 10^5 phage plaques (> 90% white) was amplified and used for gene isolation.

Library Screening. The amplified library was plated at 5 X 10^4 plaques/150 mm plate and the resulting phage particles lifted onto nylon filters (Micron Separations Inc., Westborough, MA) as described by Sambrook et al., (1989) ~~1989~~. A total of 8 filters were hybridized under conditions of high stringency (5X SSC at 68C overnight in 2% blocking reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN), followed by two 5 minute washes in 2X SSC, 0.1% SDS, followed by two 15 minute washes in 0.5X SSC, 0.1% SDS at 68C) with a 1.2 kb cDNA probe against the 48 kD i-antigen (Clark et al. Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)) labeled with digoxigenin (Genius

System; Boehringer-Mannheim Biochemicals). This probe was previously shown to encode most of the 48 kD i-antigen of parasite isolate G1. Filters were reacted with an alkaline phosphatase-tagged anti-digoxigenin antibody (Boehringer-Mannheim Biochemicals) and developed with CSPD chemiluminescent substrate (Tropix). Twelve positive clones were isolated and subjected to two rounds of plaque purification, wherein a positive clone is selected, diluted, replated and rescreened. Inserts were then subcloned into pBluescript SK(-) by *in vivo* excision in the presence R408 helper phage (Stratagene). Plasmid DNA was purified from bacterial cultures by alkaline lysis and anion-exchange chromatography using modified silicagel resins (Qiagen, Valencia, CA). Cloned inserts were mapped by digestion with appropriate restriction enzymes using techniques known to one of skill in the art (see, for instance, Sambrook et al., (1989) ~~1989~~). All positive recombinants contained a 3.3 kb *Swa* I fragment. A restriction map of the cloned insert from one recombinant (GL3-8) is shown in Fig. 6. Both strands of the region extending from the left end through the distal *Eco*R I site of the insert were subjected to nucleotide sequencing. DNA sequencing was performed with a Perkin Elmer/Applied Biosystems Division 377 automated DNA sequencer using dye terminator chemistry and AmpliTaq-FS DNA polymerase (Perkin Elmer, Norwalk, CT). Sequence analysis showed that the insert encoded the entire 48 kD i-antigen.

Transcript Mapping. The 5' end of i-antigen transcripts were mapped by primer-extension analysis. Briefly, 10 pM of antisense primer H4 (AGCAGCACCTACATCAGTCAATCC, SEQ ID NO:21) complimentary to a sequence near the putative ATG start codon of the 48 kD i-antigen gene (nucleotides +94-117), was end-labeled with $\gamma^{32}P$ (Amersham, Arlington Heights, IL) in the presence of T4 polynucleotide kinase (10 units; Promega) (Sambrook et al., (1989) ~~1989~~). The labeled primer (2 pM) was hybridized with 10 µg total RNA from *I. multifiliis* theronts (G1 isolate) and extended at 42° for 1.5 hours in the presence of AMV reverse transcriptase (26 Units; Boehringer-Mannheim). Mussel glycogen was added as a carrier (200 µg/ml; Boehringer-

Mannheim) and the extension product was precipitated by addition of 0.5 volume 7.5 M ammonium acetate and 2.5 volumes ethanol. Following centrifugation (14,000 X g for 10 minutes), the pellet was air dried and dissolved in H_20. The size of primer extension products was determined relative to a ^{32}P-labeled sequencing ladder run on parallel lanes of a 6% polyacrylamide/urea sequencing gel. The ladder was prepared in a dideoxy sequencing reaction (Sequenase kit; United Sates Biochemicals) using single-stranded M13mp18 DNA as a template, and M13 universal primer GTAAAACGACGGCCAGT (SEQ ID NO:22) labeled with ^{32}P. After electrophoresis, gels were fixed, dried and autoradiographed for visualization of labeled DNA fragments (Sambrook et al., (1989) ~~1989~~).

The 3' ends of i-antigen transcripts were mapped using the RACE (rapid amplification of cDNA ends) protocol (M. Frohman, ~~In RACE: Rapid amplification of cDNA ends. In: PCR Protocols~~: A Guide to Methods and Applications, Innis, M. A., et al., (eds.) Academic Press, San Diego, pp. 28-38 (1990)). Total RNA was prepared from *I. multifiliis* (G1) theronts by lysis in guanidine thiocyanate, and poly(A)$^+$ mRNA was purified by two rounds of chromatography on oligo(dT)-cellulose (Clark et al. Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)). First-strand cDNA was then prepared from 1 µg poly(A)$^+$ RNA, using 25 pmol of primer EPBdT$_{18}$ (GCGAATTCTGCAGGATCCAAACT$_{18}$, SEQ ID NO:23); kindly provided by Dr. Royal McGraw, University of Georgia), and AMV reverse transcriptase (0.25 unit/µl; Boehringer-Mannheim) (Sambrook et al., (1989) ~~1989~~). Following incubation at 42°C for 2 hours, a fraction of the first-strand product was used as template in a second-round PCR reaction containing either of two forward primers, namely, ICH5 (GTGTCGACAGCAGGTACTGATACATG, SEQ ID NO:24) or H5 (CGAAAACAGTGGTGGTAGTACCTT, SEQ ID NO:25) in combination with the reverse primer, EPB (GCGAATTCTGCAGGATCCAAAC, SEQ ID NO:26). The ICH5 primer corresponded to a region of the gene that lay proximal to the breakpoint between the gene and 1.2 kb cDNA sequence, while H5 corresponded to a region of the

1.2 kb cDNA that lay distal to that site. PCR was carried out under standard conditions using 20 pmol primer/100 µl reaction and 5 U *Taq* DNA polymerase (Promega) (1 minute at 94°C, 1 minute at 52°C, 1 minute at 72°C; 30 cycles). The ICH5/EPB product was electrophoresed on a 1.4% agarose gel and ran as a broad band of ~295-375 bp. DNA was eluted from the gel, digested with *Sal* I and *Eco*R I, and then directionally cloned into pBluescript SK(-) for subsequent sequence analysis.

cDNA Clones. A λZAP II cDNA library prepared from *I. multifiliis* trophont RNA was screened with a ^{32}P-labeled 24_{mer} oligonucleotide (5'-AGCAGCACCA ACATCAGTCA AACC, SEQ ID NO:27) encoding eight amino acids near the N-terminus of the 48 kD i-antigen, as previously described (Clark et al. Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)). In addition to a clone that provided the 1.2 kb i-antigen cDNA (designated 2-3), the library screen yielded several additional positive recombinants. Two such clones (designated 1-1 and 1-3) were chosen for sequence analysis. cDNA inserts were subcloned into pBluescript SK(-), and plasmid DNA sequenced on the positive strand using the ICH5 primer.

Southern blotting analysis. 5' and 3'-specific probes were generated using the polymerase chain reaction. The 5'-specific probe was a 475 bp fragment spanning nucleotides +188-662 of the gene and was amplified using the 1.2 kb cDNA as a template in conjunction with the forward and reverse primers, ATGGTAATTAACCTTTCGCAGCAAATAA (SEQ ID NO:28) and GGTCTGCATTTAACACATAA (SEQ ID NO:29), respectively. The 3'-specific probe was a 495 bp fragment amplified from genomic DNA using H5 as the forward primer, and the reverse primer AGATACATCAGTATACGAAA (SEQ ID NO:30). The later sequence was derived from the H5/EPB RACE product. Probes were purified by agarose gel electrophoresis, and labeled using random oligonucleotide synthesis (High Prime DNA labelling kit™; Boehringer-Mannheim) in the presence of α^{32}P-dCTP (Amersham). Genomic DNA (5 µg) from *I. multifiliis* theronts (G1 isolate) was digested with either *Eco*R I, *Hind* III or *Swa* I, fractionated on a 0.7% agarose gel and transferred to

nylon. The filter was then hybridized with probes specific for either the 5' or 3' ends of the 1.2 kb cDNA in separate reactions. The probes were radiolabeled to 10^9 cpm/μg andused at 10^6 cpm/mL. Hybridization was carried out under conditions of high stringency (washed overnight in 6X SSC, 10X Denhardt's reagent, 0.1% SDS, 10 μg/mL denatured herring sperm DNA at 65C; followed by one 15 minute wash at room temperature in 2X SSC, 0.1% SDS, followed by one 15 minute wash at 65C in 2X SSC, 0.1% SDS, followed by one 15 minute wash at 65C in 0.5X SSC, 0.1% SDS, followed by one 15 minute wash at 65C in 0.2X SSC, 0.1% SDS) as previously described (Clark et al. Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)).

Comparisons between gene and cDNA sequences. Results of nucleotide sequencing reactions were complied and analyzed using *DNASIS* software (version 2.0; Hitachi Software Engineering, Yokahama, Japan). When the nucleotide sequence of the 1.2 kb cDNA used as a probe in this study was compared with the coding region of the 48 kD i-antigen gene, a number of differences were observed. These included two single-base substitutions (nucleotides +781 and 952), as well as complete divergence in the region distal to nucleotide +1227 (nucleotide 1172 of the cDNA). To determine whether these differences were real, the 1.2 kb i-antigen cDNA was resequenced and found an error at position +781 (position 726 of the cDNA should read A rather than C as originally reported) (GenBank accession no. M92907 updated). The transversion at nucleotide 952, and the divergent sequence at the 3' end of the cDNA were, nevertheless, confirmed.

As indicated in Fig. 7, these differences would result in an amino acid substitution at position 318 (phenylalanine for leucine), and an entirely different sequence at the carboxy-terminus of the gene product. Specifically, the cDNA product would lack the C-terminal hydrophobic amino acid residues specified by the gene. Hydrophobic domains at the C-termini of GPI-anchored proteins are necessary for covalent attachment of glycosylphosphatidylinositol moieties (P. Englund, Annu. Rev. Biochem., 62:121-138 (1993)), and the deletion of relevant coding sequences from transgenes that encode such proteins usually results in

secretion rather than membrane binding. Furthermore, there are a number of instances in which alternative splicing of endogenous mRNAs gives rise to transcripts that either specify, or fail to encode hydrophobic C-terminal peptides (I. Caras et al., Nature, 325:545-548 (1987); H. Gower et al., Cell, 55:955-964(1988)). In the first case, such products are retained at the plasma membrane (as GPI-anchored proteins), while in the second, they are exported from the cell. Both secreted and membrane bound forms of a 48 kDa i-antigen have been described in *I. multifiliis* (C. Xu et al., J. Euk. Microbiol., 42:558-564 (1995)). It should also be noted that the parasite isolate designated G1 contains a second i-antigen gene believed to encode an antigenically related 60 kDa protein (T. Clark et al., Annu. Rev. Fish Dis., 5:113-131 (1995)); this gene is recognized by the 5'-specific probe used in this study and appears as a 5 kb fragment in the *Swa* I digest as discussed below. The 3'-specific probe fails to recognize this fragment indicating that the 1.2 kb cDNA is not a product of this gene.

Interestingly, when the 3'-end of i-antigen transcripts were mapped by RACE using a sense primer upstream of nucleotide +1227 (primer ICH5), the resulting PCR product had essentially the same sequence as the gene (Fig. 6). Consistent with this observation, two independent i-antigen cDNAs were isolated from the same library used to prepare the 1.2 kb probe and found that these contained the same sequence as the gene at their 3' ends (Fig. 6). By contrast, when a sense primer corresponding to the 1.2 kb cDNA downstream of nucleotide 1172 (primer H5; see herein) was used, the RACE product that was generated was much larger than expected (~600 bp) and had a sequence entirely different from that of the gene. Furthermore, it was not possible to generate PCR products with genomic DNA as a template in standard reactions using sense and antisense primer pairs that flanked nucleotide 1172 of the 1.2 kb cDNA.

Since this suggested that the sequences on either side of nucleotide 1172 were discontinuous within the genome, Southern hybridization analysis was carried out using probes described above corresponding to 5' and 3' regions of the 1.2 cDNA. The restriction fragments recognized by the two probes were

different. A 3.3 kb band recognized by the 5'-specific probe in the *Swa* I digest corresponded to the genomic DNA fragment described herein. A larger (5 kb) band was also observed, which may represent the gene for a related 60 kD i-antigen expressed by the G1 isolate (Clark et al., Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992); T.Clark et al., Annu. Rev. Fish Dis., 5:113-131 (1995)).

Example 2

Identification of the Open Reading Frame and Analysis of the Deduced 48 kD I-antigen Sequence from *I. multifiliis* (G1 Isolate)

Computer-based sequence analysis. Homology searches were performed at the ISREC World Wide Web server (Swiss Institute for Experimental Cancer Research) using the BLAST network service (BLAST, basic local alignment search tool; WU-BLAST server version 2.0a13) and the SwissProt+Trembl+TrUpdates peptide sequence databases. Predictions of potential signal peptides and their cleavage sites were made using the Signalp World Wide Web server (http://www.cbs.dtu.dk/services/SignalP/) version 1.0. Peptide mass was determined using the PEPTIDE MASS tool accessed through the ExPASy molecular biology World Wide Web server of the Swiss Institute of Bioinformatics (http://expasy.hcuge.ch/sprot/peptide-mass.html). Amino acid sequence alignment was carried out using the CLUSTALW (1.74) multiple sequence alignment program accessed through the ExPASy web server.

Identification of the open reading frame. The cDNA used to screen the genomic library in Example 1 begins with a 5'-terminal G that lies several nucleotides upstream of a valine codon (GTT) marking the N-terminus of the mature 48 kD i-antigen (Clark et al., Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)). This G residue corresponds to nucleotide +56 of the genomic DNA sequence shown in Fig. 1. Translating downstream from this nucleotide (and in-frame with the original cDNA), the gene was found to contain a single, uninterrupted reading frame extending through nucleotide +1326, followed by

two adjacent TGA stop codons. *I. multifiliis*, along with other hymenostomatid ciliates, utilize TGA as the only stop codon; the standard TAA and TAG triplets specify glutamine instead.

Translating in the opposite direction, the region immediately upstream of the 5'-terminus of the cDNA was found to encode a methionine (predicted by the ATG triplet at nucleotides +1-3), followed by a stretch of 19 mostly hydrophobic amino acids. The hydrophobic nature of these amino acids (along with the fact that the i-antigens are membrane polypeptides) would suggest that this region specifies a signal peptide that targets the protein to the plasma membrane. To investigate this further, neural network algorithms trained on signal peptides and their cleavage sites (H. Nielsen et al., ~~Prot. Engin., 10:1-6 (1997)~~ Prot. Engin 10:1-15 (1997)) were used to examine the first 50 amino acids of the deduced protein sequence beginning with the methionine residue cited above. Such algorithms identified the first 20 amino acids as a signal peptide (S mean = 0.839), and predicted a cleavage site between the alanine and valine residues (amino acids 20 and 21, respectively) of the deduced amino acid sequence. The N-terminal amino acid of the 48 kD antigen protein corresponds to the valine residue predicted above (Clark et al., Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992)). Taken together, these observations argue strongly that amino acids 1-20 of the deduced protein constitute a signal peptide.

Assignment of the methionine residue (amino acid position 1) as the start site of the 48 kD protein is also supported by the results of primer extension analysis on polyA$^+$ RNA from the infective theronts. I-antigen transcripts appear to initiate at two sites located -33 and -34 nucleotides upstream of the A nucleotide of the methionine codon. Because no other ATG triplets are predicted within this region, the methionine residue at position 1 almost certainly represents the translational start site of the protein itself. Based on these considerations, the coding region of the gene extends 1326 nucleotides and specifies a protein precursor of 442 amino acids having a theoretical MW_r (molecular mass) of 45,025 daltons. The gene is designated *IAG48[G1]*. A total of 23 UAA and UAG triplets (encoding glutamine in *I. multifiliis* but

functioning as stop codons in most other eukaryotes and prokaryotes) are present within *IAG48[G1]*.

Curiously, the third position of the codons shared a strong preference for either A or T (84.9%). While the significance of this is not understood, such bias appears to be a common feature of i-antigen genes (D. Martindale, J. Protozool., 36:29-34 (1989); J. Deak et al., Gene, 164:163-166 (1995)) and may reflect important constraints on RNA structure. The polyA tract at the 3' end of the RACE product, shown in Fig. 7, most likely represents the site at which polyadenylation of *IAG48[G1]* mRNA occurs. Based on this (and assuming an average length of 100-200 nt for the polyA tail), the predicted size of RNA transcripts from this gene would be in the range of 1,543-1643 nt.

Amino acid sequence motifs. Analysis of the deduced protein sequence of the gene revealed a minor discrepancy between the predicted mass of the preprocessed protein (45 kDa), and the size of the mature i-antigen based on SDS-PAGE (48 kDa) (T. Clark et al., Annu. Rev. Fish Dis., 5:113-131 (1995)). Potential secondary modifications could appreciably alter the electrophoretic mobility of the processed protein. Furthermore, structural anomalies associated with repetitive sequence motifs could lead to incorrect estimates of size based on SDS-PAGE.

This aside, the most interesting structural features of the deduced protein are hydrophobic regions at the N- and C- terminus, a consensus P-loop domain, and tandemly repetitive cysteine-rich motifs. A stretch of 14 mostly hydrophobic amino acids separated from a short spacer from three small amino acids (-Cys-Ala-Ser-) was predicted at the extreme carboxy-terminus of the protein (Fig. 1). This type of sequence is highly characteristic of an addition site for a glycosylphosphatidylinositol (GPI) anchor (P. Englund, Annu. Rev. Biochem., 62:121-138 (1993)). Other evidence shows that the 48 kD i-antigen is, in fact, GPI-anchored. The hydrophobic region at the amino-terminus of the protein is consistent with i-antigens being membrane associated proteins; presumably, the N-terminus targets the protein to the endoplasmic reticulum. The P-loop domain at position 316-323 [Gly-[Xaa_4]-Gly-Lys-Ser] (SEQ ID

NO:34) may or may not be significant. While this type of structure is generally associated with proteins that bind ATP or GTP, the presence of such motifs does not insure a role in nucleotide binding (M. Saraste et al., Trends Biochem. Sci., 15:430-434 (1990)). Indeed, proteins with closest similarity to the 48 kDa i-antigen in terms of primary structure (i.e., the *Lembadion* L-factor, *Tetrahymena* SerH, and *Giardia* VSP surface antigens) all lack a consensus phosphate-binding loop. Instead, these proteins, like the deduced 48 kD i-antigen, have numerous Cys-$Xaa_{2,3}$-Cys (SEQ ID NO:31, 32) motifs embedded within higher order tandemly repeated amino acid sequence domains.

Repetitive sequence domains. Beginning with a cysteine residue at position 23, five homologous segments with an average of 80 amino acids each were identified within the deduced 48 kD i-antigen protein sequence (Fig. 5(a)). Adjacent repeats had the greatest degree of homology, with the second and third being ~ 90% identical. As shown in Fig. 5(a) and (b), the repeats are characterized by 6 invariant cysteines that fall into register when the segments are aligned. The predominant spacing between cysteines has the order Cys-$Xaa_{2,3}$-Cys (SEQ ID NO:31, 32). There are 14 such motifs in the 48 kD i-antigen protein sequence, as well as 4 larger elements having the order, Cys-Xaa_2-Cys-Xaa_{20}-Cys-Xaa_3-Cys-Xaa_{20}-Cys-Xaa_2-Cys (SEQ ID NO:34). The spacing between the last cysteine of each repeat, and the first cysteine of the next repeat is Cys-Xaa_3-Cys (SEQ ID NO:32).

Cysteine motifs with the order Cys-$Xaa_{2,3}$-Cys (SEQ ID NO:31, 32) are common to a large and diverse family of proteins that bind zinc and other metal ions (J. Berg et al., Science, 271:1081-1085 (1996)). A search of the SWISS-PROT/TrEMBL database showed similarities (smallest sum probability level ≥ 2.8e-06) between the 48 kD i-antigen and several other entries, five of which were protozoan membrane proteins. These included the SerH immobilization antigen of the free-living ciliate *Tetrahymena thermophila* (accession no. Q27197), a putative membrane protein (L-factor) from the predatory ciliate *Lembadion bullinum* (accession no. Q94589), and three variant-specific surface proteins (VSPs) of the mammalian gut parasite, *Giardia lamblia* (accession nos.

Q24977; Q24970; P21849). Homologies with two additional entries (an antifreeze protein from Arctic cod [accession no. Q13028], and the product of an unidentified gene from *Caenorhabditis elegans* [accession no. Q17084]) were considered less significant because of unusual bias in the amino acid composition of these proteins. With regard to the protozoan membrane proteins, all contained numerous Cys-$Xaa_{2,3}$-Cys (SEQ ID NO:31, 32) motifs. The product of the *Giardia* vspA6-S1 gene (accession no. Q24970) showed the greatest similarity to the 48 kD i-antigen in terms of the overall spacing of these motifs, with 29 of a possible 30 cysteine residues in the *I. multifiliis* protein overlapping with the identical amino acid in the vspA6-S1 gene product (Fig. 8). This region has eight Cys-$Xaa_{2,3}$-Cys (SEQ ID NO:31, 32) motifs that do not align with similar domains in the *I. multifiliis* polypeptide.

Example 3

Identification of an *I. multifiliis* (G5 Isolate) Gene Encoding a 55 kD I-antigen

<u>Construction of G5 cDNA library</u>. A G5 cDNA library was made as follows. First-strand cDNA synthesis was primed with a 20-fold molar excess of oligodT_{18} in a reaction containing 5 μg of polyA^+ RNA from *I. multifiliis* theronts (strain G5) and AMV reverse transcriptase as described by Krug et al. (<u>Meth. Enzymol</u>.152:316-325 (1987)). Second-strand synthesis was carried out in the presence of DNA polymerase I and Rnase H, followed by hairpin loop cleavage with mung bean nuclease as described by Gubler (<u>Meth. Enzymol</u>.152:330-335 (1987)). The second-strand product was size-fractionated and adaptors were added as in construction of the G1 genomic DNA library (Example 1). After removal of excess adaptors, material > 500 bp was cloned into the lambda phage vector λZAPII™ as for G1 genomic DNA.

<u>Screening of the G5 cDNA library</u>. The G5 cDNA library was first screened under conditions of reduced stringency with a digoxigenin-labelled probe corresponding to the coding region of the 48 kD i-antigen gene of parasite

isolate G1. No positive clones were identified. Previous attempts to screen a custom-made genomic DNA library from another parasite isolate (G1.1) with the same probe had also resulted on no positive clones.

Degenerate PCR primers were then produced against the 5' and 3' ends of the 48 kD i-antigen gene. These primers, designated #35 and #37, corresponded to the 5' and 3' regions of the coding sequence of *IAG48[G1]*, and the primer sequences were as follows: #35: ATGAAATA(C/T)AA(C/T)ATTTTATTAATT (SEQ ID NO:35), which is a 4-fold degenerate sense primer corresponding to all or part of the amino acid sequence MKYNILLT (SEQ ID NO:36) at the N-terminus of the *IAG48[G1]* gene product; and #37: AAATAATAA(G / A)GAAAT(A / C)GATAAAAA (SEQ ID NO:37), which is a 4-fold degenerate antisense primer corresponding to all or part of the amino acid sequence FLSISLLF (SEQ ID NO:38) at the C-terminus of the *IAG48[G1]* gene product. PCR conditions were 30 cycles of 94° C for 1 minute, 52° C for 1 minute, 72° C, for 1 minute. A single band corresponding to an amplified i-antigen coding region (55 kD) was expected. However, this reaction yielded several bands on agarose gels visible by ethidium staining, none of which had the expected size of a product from a gene encoding a 55 kD protein (Fig. 9).

To determine which band, if any, corresponded to an amplified i-antigen coding region, the amplification products were Southern blotted and probed under conditions of reduced stringency with a radiolabelled cDNA probe corresponding to the 48 kD i-antigen of isolate, i.e., radiolabelled *IAG48[G1]*. Hybridization conditions were 6X SSC (Denhardt's, SDS, herring sperm DNA) at 55° C overnight. Blots were washed in 2X SSC at RT (2 X 15 minutes), followed by 2X SSC at 55° C (2 X 15 minutes). The *IAG48[Gl]* probe revealed a band of about 1.1 kb on the blot.

The amplification products were again resolved by agarose gel electrophoresis, the region of the gel corresponding to DNA fragments of approximately 1.1 kb was excised, and the fragment was labeled with ^{32}P-dCTP. The radiolabelled fragment was used to probe the G5 cDNA library under high stringency conditions as for the G1 genomic DNA library (Example 1). A single

positive clone containing a 1 kb cDNA insert was identified. On sequencing, this cDNA predicted a protein with all the hallmarks of an i-antigen, *viz.* tandemly repetitive amino acid sequence domains (approximately 80 amino acids each) containing 6 periodic cysteine residues (C-$X_{2,3}$-C motifs), in addition to a stretch of hydrophobic amino acids at the C-terminus virtually identical to those predicted by the gene for the 48 kD antigen. The 1 kb cDNA was nevertheless truncated and lacked the region coding for the N-terminal part of the protein.

RACE (Rapid Amplification of cDNA Ends) was carried out in an attempt to determine the missing 5' sequence. An antisense primer designated G5-11 (TGCTCGAGAATCTGTTGCTCCACCTG, SEQ ID NO:39) was used for first-strand cDNA synthesis, then a polyG tail was added and the resulting cDNA was amplified with the forward and reverse primers Q_o (CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCCCCCCCCCCCC CCCCCC, SEQ ID NO:40) and, G5-11 (TGCTCGAGAATCTGTTGCTCCACCTG, (SEQ ID NO:39) respectively (PCR conditions: 1 cycle of 94° C for 3 minutes, 30° C for 2 minutes, 72° C for 40 minutes, then 30 cycles of 94° C for 45 seconds, 53° C for 45 seconds, 72° C for 90 seconds (e.g., Frohman, Meth. Enzymol. 218:340-356 (1993)). This was followed by a second round of (nested) PCR using the forward and reverse primers Q_1 (GAGGACTCGAGCTCAAGC, SEQ ID NO:41) and G5-12 (AACTCGAGTACCAGCAGGGCATTTAAC, SEQ ID NO:42), respectively (PCR conditions: 30 cycles of 94° C for 45 seconds, 53° C for 45 seconds, 72° C for 2 minutes). This produced a RACE product that approached the extreme 5' end of the coding region of the G5 gene *(IAG55(G5)),* but still failed to reach it; no ATG start codon was observed.

We then resorted to a procedure referred to as inverse PCR (see Fig. 10). Based on the known sequence of the 1 kb cDNA (the shaded bar in Fig. 10), two oppositely oriented primers, G5-11 (SEQ ID NO:39) and G5-4 (CACACCTTGTCCGGCAATTAAAC, SEQ ID NO:43) were designed and used to amplify the regions flanking the 5'- and 3'-ends of the 1 kb cDNA.

Genomic DNA (1 μg) from the G5 parasite isolate was incubated with a variety of different restriction enzymes (including Afl II and Swa I) in separate 20 μl reactions for 6 hours. DNA fragments were brought to a volume of 200 μl in 1X DNA ligation buffer (Gibco-BRL Life Technologies, Gaithersburg, MD), then chloroform extracted and allowed to self-anneal at 22° C for 12 hours in the presence of T4 DNA ligase (40U) (Gibco-BRL). DNA was ethanol precipitated and amplified using the forward and reverse primer pair G5-11 and G5-4 under the following conditions: 10 cycles of 94° C for 10 seconds, 60° C for 30 seconds, 68° C for 4 minutes, followed by 20 cycles of 94° C for 10 seconds, 60° C for 30 seconds, 68° C for 4 minutes (+ 20 seconds for each cycle). DNA fragments of about 1.6 and about 1.9 kb were produced in the samples generated by the restriction enzymes Afl II and Swa I, respectively. The amplified products were cloned into the pCR-Script™ plasmid vector (Stratagene) and sequenced. Both clones contained the 5' end of the coding sequence of the 55 kDa i-antigen gene, along with additional sequences flanking the 5' and 3' ends of the coding regions.

New forward and reverse primers were constructed based upon nucleotide sequences of the 5' and 3' regions flanking the 55 kD coding region, and these primers were used to amplify the entire coding region of the gene using G5 genomic DNA as a template. A comparison between the deduced amino acid sequence encoded by the 48 kD i-antigen gene and the deduced amino acid sequence encoded by the 55 kD i-antigen gene, and of the coding regions of the two genes, is shown in Fig. 3.

Example 4

Glycolipid Anchoring of *I. multifiliis* (G5 Isolate) 55 kD I-antigen Protein

Membrane proteins from *I. multifiliis* (strain G5) were extracted with the non-ionic detergent Triton X-114. Following Triton extraction, detergent micelles (containing amphipathic proteins) were treated with a recombinant GPI-phospholipase C (GPI-PLC) from African trypanosomes which cleaves the

lipid anchor from GPI-linked membrane proteins. Fig 11, left panel, shows a Coomassie blue stained gel of theront membrane proteins fractionated by SDS-PAGE. Lane (A) contains total detergent soluble membrane proteins. The prominent band at 55 kD represents the i-antigens of the G5 strain. Lane (B) shows the detergent soluble membrane protein fraction after cleavage with GPI-PLC. Lane (C) contains proteins liberated into the aqueous phase afer enzyme treatment. Note the transfer of the 55 kD band from the detergent to the aqueous phase. Fig. 11, right panel, shows a Western Blot of fractions (A) and (C) reacted with an antibody conjugate that recognizes a cross-reacting determinant on the cleaved portion of the GPI-anchor that becomes exposed following enzyme treatment. Positive staining of a 55 kD band in lane (C) (but not A) argues strongly for GPI-linkage of *Ichthyophthirius* i-antigens with the plasma membrane.

Example 5

Recombinant Production of a 55 kD I-antigen Protein in *E. coli*

Synthesis of a synthetic gene. A synthetic G5 i-antigen gene sequence (SEQ ID NO:102, Fig. 2(b)) was constructed using a method known as DNA shuffling essentially as described by Stemmer, et al. (Gene 164:49-53 (1995)). Eighteen overlapping primers (primers 3201-3218, Fig. 12) spanning the entire length of the G5 i-antigen coding sequence were combined in a single tube at a final concentration of 5 µM and allowed to assemble in a cycling reaction carried out 30 times at 94° C for 15 seconds, 52° C for 30 seconds and 70° C for 30 seconds (plus 10 additional seconds in each cycle). Following the assembly reaction, 2.5 µl of the product was amplified with the original flanking primers (3201 and 3218) to produce a full-length synthetic gene. The resulting product was cloned and sequenced. Mutations arising from error-prone synthesis PCR were corrected by site-directed mutagenesis (Transformer Site-Directed Mutagenesis kit, Clontech Laboratories, Inc., Palo Alto, CA).

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Synthesis of modified versions of the synthetic gene. Based on its deduced sequence, the 55 kD protein contains hydrophobic sequences at its N- and C-terminus that are presumed to target the protein to the plasma membrane (the N-terminal sequence acting as a signal peptide for ER localization, and the C-terminal sequence acting as a GPI-anchor cleavage and addition site). To determine whether different gene products might be processed differentially by the immune system following their expression in fish, we made two modified versions of the gene, one lacking the GPI-anchor addition sequence and the other lacking the N-terminal signal peptide. Specifically, we wanted to determine whether the product of the C-terminal deletion might give rise to a stronger humoral immune response since it was expected be secreted from cells rather than be bound to the plasma membrane through a glycolipid anchor. Therefore, having made the synthetic gene for the 55 kD protein, we used PCR to construct the modified versions of the full-length gene. The first lacked the coding region for the signal peptide at the N-terminus (residues 1-20), and the second lacked the hydrophobic stretch at the extreme C-terminus of the protein (amino acids 453-468).

I-antigen protein production in *E. coli*. A recombinant version of the 55 kD *I. multifiliis* i-antigen protein of parasite isolate G5 (SEQ ID NO:7) was produced in *E. coli* strain XL-1 Blue using the plasmid expression vector pProEX™-1 (Gibco-BRL LifeSciences) into which a version (SEQ ID NO:53) of the synthetic gene (SEQ ID NO:5) had been inserted. This version (SEQ ID NO:53) was identical to SEQ ID NO:5 except that it contained a point mutation at nucleotide position 17 in the coding sequence (Fig.13), resulting in the substitution of a proline for a leucine at amino acid position 6 in the i-antigen protein sequence (L6P) (SEQ ID NO:54) (Fig. 14). Logarithmic cultures were incubated in IPTG to induce expression of the 55 kD antigen fused to a 6x-histidine tag and rTEV protease cleavage signal. Fig. 15 shows a Western blot of a 10% SDS-polyacrylamide gel on which equivalent amounts of bacterial protein from cells taken before (1) and above (2) induction with IPTG were run. The blot was reacted with monospecific rabbit antibodies against the 55 kD i-

antigen of the G5 strain followed by goat anti-rabbit IgG couples to alkaline phosphatase. Color substrates were NBT an BCIP. Note the strong band at about 55 kD in the induced sample.

The modified versions of the synthetic gene that lacked membrane targeting sequences at their 5'- or 3'- ends were cloned into several procaryotic expression vectors containing IPTG-inducible promoters (pProEX-1 (Gibco), pET22b(+) (Novagen), pQE-16 (Qiagen) and pGEX-4P- I (Pharmacia)). Recombinant proteins of the expected size that reacted strongly on Western blots with monospecific polyclonal antibodies against the 55 kDa antigen were produced in all bacterial transformants following the addition of IPTG.

Example 6

Immune Response of Catfish

to Vaccination with *I. multifiliis* (G5 Isolate) 55 kD I-antigen Protein

It is known that naive fish are completely protected against infection by *I. multifiliis* following passive transfer of immobilizing murine monoclonal antibodies (mAbs). To test whether *I. multifiliis* i-antigens themselves can elicit protective immunity, vaccination trials were carried out with channel catfish *(Ictalurus punctatus)* immunized with 55 kD i-antigen purified by mAb affinity chromatography from *I. multifiliis* serotype D. Fifty channel catfish (each weighing 10-15 g) were immunized by two intraperitoneal (i.p.) injections (two weeks apart) consisting of 10 μg of purified 55 kD i-antigen of the *I. multifiliis* G5 isolate (i-Ag) in Freund's complete or incomplete adjuvant. The same number of negative control animals were immunized similarly with either an irrelevant 14 kDa *I. multifiliis* G5 protein (14kD) or bovine serum albumin (BSA). A fourth group of positive control fish was vaccinated by two i.p. injections of 8000 and 10,000 live G5 parasites (live *Ich*) without adjuvant (this treatment has previously been shown to elicit protective immunity). All groups were challenged with infective G5 theronts (15,000/fish) 8 weeks after the last injection. Seventy-two percent (72%) of fish immunized with the i-antigen and

59.2% of fish immunized with live parasites survived challenge (Fig. 16). All of the negative control animals died. In addition, there were significant differences (P>0.001, Kruskal-Wallis one way ANOVA) between the median days to death of controls and the fish that died following immunization with i-antigen or live theronts.

In a separate trial, single i.p. injections of 10 μg of i-antigen mixed with either a CpG oligodeoxynucleotide (19-mer) adjuvant or Freunds complete adjuvant provided 33% and 40% protection, respectively. Control fish immunized with i-antigen given with a non-CpG oligonucleotide or BSA with a CpG oligodeoxynucleotide all died following challenge.

Serum and mucus antibody production in vaccinated fishes corresponded with protection. These results demonstrate the efficacy of purified i-antigen with adjuvant in eliciting protective immunity following vaccination.

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Example 7

Immune Response of Channel Catfish to Vaccination with *I. multifiliis* (G5 Isolate) 55kD I-antigen Plasmid Vaccine

Truncated versions of the 55kD i-antigen from *I. multifiliis* (G5 isolate) containing N-terminal and C-terminal deletions (Example 5), along with the full-length sequence, were cloned into the eucaryotic expression vector pcDNA3.1 for use fish vaccine trials. Although we have not yet determined how the respective gene products localize in cultured fish cells, an equivalent C-terminal deletion in the gene for the 48 kD i-antigen from serotype A resulted in secretion of the corresponding protein following transformation of *T. thermophila* (Example 9, Fig. 17).

Groups of 10 fish were vaccinated by intramuscular injection with either 10, 1 or 0. 1 μg of plasmid DNA (pcDNA3. 1) containing either the full-length, N-terminal or C-terminal deletion of the synthetic 55 kDa i-antigen gene. A positive control group vaccinated with the purified antigen in Freund's complete adjuvant (see Example 6), and a negative control group injected with the

pcDNA3.1 containing the lacZ gene were included in the study. Sera were withdrawn from fish at two week intervals beginning 14 days after injection and antibody levels were determined by Western blotting and ELISA.

SDS-PAGE was carried out under both reducing and non-reducing conditions, and Western blots were probed with pooled antisera from the different groups of fish. The 55 kD i-antigen from parasite isolate G5 was affinity purified and fractionated by SDS-PAGE under non-reducing conditions (50 ng/lane). Protein was then transferred to PVDF membrane and reacted sequentially with pooled sera from fish vaccinated with pcDNA3.1 DNA vaccine constructs followed by a monoclonal antibody cornjugate (alkaline phosphatase) against the heavy chain of channel catfish Ig. The Western blot is shown in Fig. 18. Lanes were probed with sera from fish immunized as follows: Lanes 1-3, 10, 1, and 0.1 μg full length gene construct, respectively; lanes, 4-6: 10, 1, and 0.1 μg C-terminal deletion construct, respectively; lanes 7-9: 10, 1, and 0.1 μg N-terminal deletion construct, respectively; lane 10, 10 μg LacZ construct (negative control); lane 11, naive fish serum; lane 12, fish vaccinated with 20 μg affinity purified 55 kDa i-antigen (positive control). Sera from fish vaccinated with constructs harboring the C-terminal deletion (lanes 4-6) reacted positively with the 55 kD protein run under non-reducing conditions, as did sera from fish injected with the purified antigen itself (lane 12). In contrast, only weak signals were seen in equivalent blots of protein separated under reducing conditions, the one exception being with sera from fish injected with 10 μg of the full-length construct. These results suggest that antibodies against the product of the C-terminal deletion (and against the i-antigen itself) are directed primarily towards conformational epitopes, while those against the full-length protein are directed towards linear epitopes. Of further interest was the fact that the antibody response to the vector encoded antigens decreased over time and was not detectable by ELISA 6 weeks after vaccination.

The animals were challenged with lethal numbers of parasites (15,000 theronts per fish) 9 weeks after immunization. The percent survival in all groups injected with the i-antigen constructs was higher than that in the negative

control (LacZ). Still, not all fish died in the LacZ control group, and because the number of fish injected was small, the relative differences between groups were statistically significant in only one case (Table 1). This experiment did not include a group of fish injected with PBS alone.

Table 1.

Immunogen	LacZ	FIO	F1	FOA	C10	C1	C0.1	N10	N1	N0.1	GSiAg
No- of fish challenged	9	9	10	10	9	10	10	9	10	10	10
No. of fish survived	4	8	8	9	6	7	8	7	10	8	10
Survival %	44.4	88.9	80	90	66.7	70	80	77.8	100	80	100
RSP	N.A.	50	44.5	50.1	33.4	57.7	44.5	42.9	55.6	44.5	55.6
*MDD ± SD	14.0 ± 4.5	17.0	14.5 ± 13A	16.0 ± 1.5	20.3 ± 5.5	12.3 ± 57	21.0 ± 6.4	10.5		21.0 ± 7.1	

Groups of fish were injected with 10, 1 or 0.1 µg pcDNA3.1 DNA containing the sequence for either the full-length 55 kD i-antigen (F10, F1, F0.1), the C-terminal deletion (C10, C1l, C0.1), or the N-terminal deletion (N10, N1, N0.1). Two additional groups of fish were injected with plasmid DNA containing a LacZ insert (LacZ), and 20 µg the affinity purified 55kD protein (i-Ag). Fish were challenged 9 weeks after vaccination with 15,000 theronts per fish.

*Mean days to death + /-Standard Deviation: no significant difference between groups by One Way Anova,

**A significant difference in percent survival was seen between the negative control group (LacZ) and the test groups N I and G5 i-Ag ($P<0.5$).

Because animals were exposed to what should have been a lethal challenge, the fact that some fish survived in the group injected with the lacZ constructs might be interesting in light of recent evidence that methylated CpG motifs in bacterial DNA can stimulate both cytokine production and B-cell proliferation, and in some cases, can protect animals non-specifically against microbial pathogens.

Example 8

Construction of a Multivalent *I. multifiliis* Vaccine

A multivalent vaccine vector is constructed based on the i-antigens of *I. multifiliis* serotype A or serotype D. The gene for the 48 kD i-antigen of serotype A has been isolated and the parasite itself is currently available for vaccine trials. If the 48 kD i-antigen is used, a synthetic copy of the gene equivalent to the one made for the 55 kD antigen (Example 5) is constructed. The construction is straightforward and involves the design and synthesis of overlapping oligonucleotides (-100 bp each) that span the coding region of the gene. The oligonucleotides are mixed at equimolar concentrations in a single reaction and allowed to assemble into the full-length sequence using a thermostable DNA polymerase (High Fidelity Expand enzyme, Roche). A fraction of the assembled product is then used as a template for amplification of the gene in a standard PCR reaction using flanking primers to drive the reaction. The resulting full-length product is gel-purified, cloned into pcDNA3.1 and sequenced. Errors in the sequence are corrected by *in vitro* mutagenesis according to the method of Deng et al. (Anal. Biochem. 200:81 (1992)) and the final version is tested as a DNA vaccine. A combined vaccine containing plasmid vectors for both the 48 and 55 kD i-antigens is also tested on fish challenged with serotype A and D.

Example 9

Immune Response of Channel Catfish to Live Vaccine: Transformed *Tetrahymena* Expressing Full-length or Truncated *I. multifiliis* (Serotype A) 48 kD I-antigen Protein and Heterologous Challenge with *I. multifiliis* (Serotype D)

The *IAG48[G1]* gene of *Ichthyophthirius multifiliis* G1 encodes the GPI anchored 48- kDa i-antigen. The extreme 3' region of the gene encodes a stretch of 14 mostly hydrophobic amino acids separated by a short spacer from three small amino acids (CAS). This sequence encodes the protein's GPI anchor addition site. *Tetrahymena thermophila* cells transformed with the entire *IAG48[G1]* gene produce an intact i-antigen anchored to the cells' surface. *Tetrahymena* cells transformed with a modified *IAG48[G1]* gene construct lacking the 3' sequence which includes the GPI addition site would be expected to produce a truncated protein lacking the GPI anchor.

Tetrahymena cells were transformed with either the gene encoding the full-length *Ichthyophthirius* G1 48- kDa i-antigen protein, or a truncated version of the gene that encodes the i-antigen protein lacking 19 amino acids at the carboxy terminus. Transformants encoding the intact or C-terminal truncated i-antigen were grown in standard *Tetrahymena* growth medium. Cell pellets and supernatant fluids were collected at the time points indicated. I-antigen was detected in cell cytosol, cell membrane or cell culture supernatants by Western blots using rabbit antisera against affinity purified *Ichthyophthirius* G5 i-antigen (see Fig. 17). It is clearly seen that the truncated protein is secreted into the culture medium.

Groups of channel catfish (6 fish per group) were immunized by bath exposure (10^6 or 10^5 cells/fish) or intraperitoneal injection with *T. thermophila* transformants (10^6, 10^5, or 10^4 cells/fish) producing intact or truncated i-antigen. A third group of fish was immunized with membrane protein extracts (1 mg or 0.1 mg/fish) from *T. thermophila* producing the full length protein.

Immunization by bath exposure to Tetrahymena transformants. Two groups of fish (6 fish in each group) were immunized by bath exposure. The fish were exposed to either 10^6 or 10^5 cells /fish for a period of 24 hours. Two immunogens were used: 1) transformed *Tetrahymena* cells expressing the entire *Ichthyophthirius* G1 48-kDa protein, and 2) transformed cells secreting a truncated form of the i-antigen lacking the GPI anchor. Fish in the control group were exposed to *Tetrahymena* transformants expressing the *neo1* gene product. Fish were exposed twice at a 30 day interval and challenged 30-60 days after the last immunization with the G5 *Ichthyophthirius* isolate. There were no significant differences (z test) between test and control groups (see Table 2). Immunized fish were challenged with a heterologous strain (G5 isolate) of *Ichthyophthirius* expressing a different i-antigen than that produced by the recombinant *Tetrahymena* used for vaccination. It is expected that challenge with a strain of *Ichthyophthirius* producing an i- antigen homologous to the G1 48 i-antigen would show increased levels of protection.

Table 2. Vaccination by bath exposure

Immunogen	Dose (cells/fish)	Number of fish challenged	Number of fish surviving	% survival	RSP[3]	MDD[4] ± SD[5]
Neo control	10^6	5	2	40	N.A.	17.0 ± 1.0
TG1[1]	10^5	5	3	60	33.3	18.5 ± 2.1
TG1	10^6	6	3	50	25.0	13.3 ± 4.9
sTG1[2]	10^5	6	3	50	25.0	21.0 ± 4.6
sTG1	10^6	6	4	66.7	40.0	17.0 ± 1.4

[1] *Tetrahymena* expressing intact membrane form of *Ichthyophthirius* G1 i-antigen.

[2] *Tetrahymena* secreting truncated form of G1 i-antigen.

[3] Relative Survival Percent = 1- (number of dead fish in test group/number of dead fish in control group) x 100%

[4] Mean days to death

[5] Standard deviation

Immunization by injection of Tetrahymena transformants. Fish in each group were injected intraperitoneally with 10^6, 10^5, or 10^4 live transformed *Tetrahymena* cells/fish. The same immunogens and controls were tested as in the immersion vaccinations. Fish were injected two times at a 30 day interval, and challenged 21 days after the last immunization with G5 *Ichthyophthirius*. A greater degree of protection was elicited in immunized fish compared to controls (Table 3).

Table 3. Vaccination by injection

Immunogen	Dose (cells/fish)	Number of fish challenged	Number of fish surviving	% survival	RSP[3]	MDD[4] ± SD[5]
Neo control	10^5	6	2	33.3	N.A.	15.3 ± 3.6
TG1[1]	10^6	5	3	60	44.5	19.0 ± 2.8
TG1	10^5	5	4	80	58.4	15.0 ± 0.0
TG1	10^4	6	2	33.3	0	14.0 ± 1.4
sTG1[2]	10^6	6	5	83.3	50.0	21.0 ± 4.6
sTG1	10^5	6	3	50.0	25.0	20.0 ± 5.7

[1] *Tetrahymena* expressing intact membrane form of *Ichthyophthirius* G1 i-antigen.

[2] *Tetrahymena* secreting truncated form of G1 i-antigen.

[3] Relative Survival Percent = 1- (number of dead fish in test group/number of dead fish in control group) x 100%

[4] Mean days to death

[5] Standard deviation

Serum antibody production. Fish serum antibody responses against recombinant G1 *Ichthyophthirius* i-antigen were determined by ELISA at 2, 4, and 6 weeks after immunization. Serum antibodies from immunized fish were detected with a sandwich ELISA technique that used wells coated with a cross-reactive rabbit antibody against *Ichthyophthirius* G5 i-antigen to capture recombinant G1 i- antigen produced in transformed *Tetrahymena*. Sera from test and control fish were added to wells and antibodies that bound to the captured i-antigen were detected using an alkaline phosphatase labeled mouse mAb against the immunoglobulin heavy chain of channel catfish. ELISA controls consisted of antibody-coated wells reacted with membrane protein from *Tetrahymena* cells transformed with the *neo1* gene.

Fish injected with *Tetrahymena* membrane protein produced high levels of serum antibody against the recombinant i-antigen. The antibody response elicited by fish immunized with live cells was almost an order of magnitude lower. The antibody response of fish immunized by bath or i.p. injection with live cells secreting recombinant i-antigen was approximately two-fold greater than the antibody response of fish immunized with *Tetrahymena* producing the membrane-bound, intact i-antigen. In Fig. 19, the differences in antibody production between fish immunized with the (a) membrane associated or (b) secreted form of the i-antigen are shown. These results suggest that live cells secreting antigen are more efficatious in eliciting the production of serum antibodies. The mucosal antibody response was not determined in these experiments.

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Example 10

Immune Response of Channel Catfish to Live Vaccine: Transformed *Tetrahymena* Expressing Full-length or Truncated *I. multifiliis* (Serotype A) 48 kD I-antigen Protein and Homologous Challenge with *I. multifiliis* (Serotype A)

Tetrahymena cells were transformed with either the entire *Ichthyophthirius* G1 48- kDa i-antigen protein, or a truncated gene sequence which encodes the i-antigen protein lacking 19 amino acids at the carboxy terminus as in Example 9.

Groups of channel catfish (70 fish per group) were vaccinated by intraperitoneal injection with 10^6 *T. thermophila* transformants producing intact or truncated i-antigen. A third group of fish (control group) was vaccinated with *T. thermophila* transformants expressing *neo*. No adjuvant was used in any of the vaccinations. The fish were boosted 2 weeks following the initial injection and bled at 3 weeks following the initial injection. Sera from 3 fish per group were pooled.

A 96 well ELISA plate was seeded with a homologous strain (i.e., serotype A) of *I. multifiliis* (strain NY1, a G1 isolate), 200 cells per well. Fish sera were serially diluted and added to the wells, and the effect on the motility of *I. multifiliis* was observed. Immobilization of *I. multifiliis* was immediately evident at serum dilutions of 1:20, and at higher concentrations the organisms exhibited clumping (Fig. 20). Sera from the control group did not cause any change in motility of *I. multifiliis*.

As another control, additional wells were seeded with a heterologous strain of *I. multifiliis* (a G5 isolate). The motility of these organisms was not affected by sera from any of the groups of vaccinated fish, confirming that the immobilization epitopes on *I. multifiliis* i-antigens are highly specific.

For comparison, two other groups of fish were vaccinated with purified subunit proteins produced from recombinant *Tetrahymena* (either the full-length 48 kD i-antigen protein or the C-terminal truncated version). The subunit proteins were adjuvanted with Freund's Complete Adjuvant. In a plate assay similar to the one described above using the homologous strain of *I. multifiliis*, some immobilization was observed but not to the degree caused by the "live vaccine." This observation lends support to the expectation that the "live vaccine" will prove to be more efficacious than the analogous protein subunit vaccine.

Example 11

Degenerate Primers for Amplification of I-antigen Genes from Other Serotypes

Forward and reverse primers for amplifying i-antigen gene sequences from different parasite isolates were designed based on regions of homology discovered between the 48 kDa (G1 strain) and 55 kDa i-antigens (G5 strain) of *I. multifiliis*. Forward primer P2 was successfully used to amplify sequences from a variety of different parasite isolates in combination with the reverse primers P3, P4 and P5. Primer sequences are as follows: P2:

CCGAATTCTCTGG(C/T)ACTGCACTTGATGATGGAG (SEQ ID NO:45), which is 2-fold degenerate and corresponds to all or part of the amino acid sequence GTALDDGV (SEQ ID NO:46); it contains an EcoR I restriction site for cloning purposes; P3: GTGGATCCAGTACATGTTACA(A/G)TACCTGC (SEQ ID NO:47), which is 2-fold degenerate and corresponds to all or part of the amino acid sequence AGTDTCT (SEQ ID NO:48); it contains a BamH I restriction site for cloning purposes; P4: GTGGATCC(A/G)CCAGAAGTTAATTTTTTA(T/G)TAC (SEQ ID NO:49), which is 4-fold degenerate and corresponds to all or part of the amino acid sequence CTKKLTSGA (SEQ ID NO:50); it contains a BamH I site; and P5: GTGGATCCAAGGAAAT(C/T)GATAAAAA(T/A)TTAGCG (SEQ ID NO:51), which is 4-fold degenerate and corresponds to all or part of amino acid sequence FAKFLSISL (SEQ ID NO:52); it contains a BamH I restriction site. PCR amplification of i-antigen gene sequences was carried out under standard conditions (30 cycles at 94° C for 1 minute, 52° C for 1 minute ,72° C for 1 minute).

Fig. 21 shows PCR amplification of genomic DNA from several serotypic variants using the P2/P4 primer pair. Amplification products were run on 1.5% agarose gel and stained with ethidium bromide. Multiple bands seen in some of the lanes may be due to amplification from more than a single i-antigen gene (serotype A, for example, has two i-antigen genes) or to mispriming from closely related sequences within tandem repeats of the same gene. The major band in the lane containing G5 isolate DNA is precisely the expected size (~800 bp) based on the sequence of the 55 kD i-antigen gene. Among the ten isolates represented in Fig. 21, two belong to serotype A (G1 and G10) and four to serotype D (G3, US, G7, and JI). Isolates G1.1, G2 and 04 correspond to serotypes B, C and E, respectively (the CUl isolate has not yet been typed).

Example 12

Construction of an I-antigen/C3d DNA Vaccine

Several distinct genes for the third component of trout complement have been identified. Although unique from one another, they share the same coding region for C3d (J. Sunyer et al., Proc. Natl. Acad. Sci USA 93, 8546 (1996)). Preparation of the hybrid i-antigen/C3d construct is carried out as follows. Three separate PCR reactions are performed to synthesize the basic elements of the construct. The first reaction involves amplification of the 55 kDa i-antigen gene using a forward primer that encodes restriction endonuclease cloning sites and replaces the existing N-terminal signal peptide of the i-antigen with the signal peptide of trout Ig. The reverse primer in this case contains restriction sites as well, and omits the coding sequence for the last 15 amino acids at the C-terminus of the protein (ordinarily, the C-terminus acts as a GPI-anchor addition site). The second PCR reaction involves synthesis of the C3d fragment containing appropriate restriction sites at its ends. The forward primer in this case mutates the first cysteine in the C3d fragment to a serine (thereby assuring correct disulfide bridging between cysteine residues within C3d.. The final PCR reaction involves synthesis of a second C3d fragment containing a short linker sequence ($GS[G_4S]_2$) (SEQ ID NO:101) at its C-terminus along with appropriate restriction sites that allow cloning between the i-antigen sequence and the first C3d PCR product. The template for synthesis of the C3d fragments is trout genomic DNA. The entire construct is assembled by first cloning the PCR product for the i-antigen gene into pcDNA3.1. The C3d products are inserted at the 3' end of the i-antigen gene using restriction sites built into the fragments. Following transformation of bacteria with the resulting construct, the vector is directly sequenced to determine the number of C3d fragments in the hybrid, as well as the accuracy of the overall sequence. The same basic strategy is employed in the construction of a HEL (hen egg-white lysozyme)/C3d hybrid control vaccine. The rationale for including the model antigen (HEL) in these studies relates to the uncertainty associated with correct folding of the i-

antigen/C3d hybrid (and consequently the likelihood that it will induce the expected result). After correcting nucleotide sequence errors, groups of fish (in this case 20-50 g rainbow trout) are injected with varying amounts of either the i-antigen or HEL hybrid constructs and the antibody response is determined in plate ELISAs (as described in other Examples) using secondary antibody conjugates against trout Ig. Trout immunized with the i-antigen/C3d hybrid vaccine are also challenged with parasites of the G5 isolate to determine levels of protection.

Example 13

Oral Delivery of a DNA Vaccine

Oral delivery represents an extremely attractive alternative for administration of DNA vaccines, particularly in aquaculture species. Based on recent experiments in mice (D. Jones et al., Dev. Biol. Stand. 92:149 (1998); K. Roy et al., ~~Nature Med. 3:387~~ Nature Med. 5:387-391 (1999)) it may now be possible to administer genetic vaccines orally using biodegradable polymers. Fish are expected to be capable of developing cutaneous mucosal antibody responses against *Ichthyophthirius* following oral vaccination with vector-encoded antigens.

Juvenile channel catfish are vaccinated orally using i-antigen based DNA vaccines complexed with chitosan. Encapsulation is performed substantially in accordance with the method of K. Leong et al., (J. Controlled Release 53:183 (1998)). Briefly, chitosan (MW 390,000) is dissolved at a concentration of 0.02% in 25 mM sodium acetate, pH 5-7. After prewarming to 55°C, 100 µl aliquots are added to an equivalent volume of vector DNA (1 ug/ml) in sodium sulfate buffer at 55°C, and the mixture is vortexed rapidly for 20 seconds. Nanoparticles formed by complex coacervation are examined by scanning electron microscopy and the resulting preparations stored at room temperature. Preparations are combined with fish food and animals are fed the equivalent of 10- 100 µg plasmid. DNA. Antigen-specific antibody responses are determined

in cutaneous mucus and serum by ELISA, and positive fish are challenged with live theronts.

The complete disclosures of all patents, patent applications, publications, and nucleic acid and protein database entries, including for example GenBank accession numbers and EMBL accession numbers, that are cited herein are hereby incorporated by reference as if individually incorporated. Various modifications and alterations of this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not to be unduly limited to the illustrative embodiments set forth herein.

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